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FROM THE DEPARTMENT OF SEROLOGY AND BACTERIOLOGY AND FROM THE WOMEN'S CLINIC, UNIVERSITY OF TURKU

THE SPERMICIDAL EFFECT IN VITRO OF SOME CONTRACEPTIVES

by

AIRI LEIKKOLA and M. E. TAKALA

(Received for publication January 3, 1952)

The principal contraceptive means used for birth control, as far as the woman is concerned, are vaginal pessaries, and in combination with these some chemical, acid jelly, or only spermicidal chemicals. The chemical is used mostly in the form of acid jelly, an oval, a tablet, or a vaginal suppository, the basic element of which has owing to its low surface tension the property of spreading rapidly in the vagina. There are now plenty of such preparations for sale. Since no publication has appeared in Finland concerning their effect and since they are being prescribed especially at marriage instruction, we have considered it worth while to investigate the spermicidal power of these substances *in vitro*.

All the preparations were obtained from the retail stores and kept at room temperature during the period of testing. For the sake of control a contraceptive preparation which had proved most ineffective was also tested, and this was obtained as fresh as possible from the chemist's.

The acid jellies tested were the American *Ortho-Gynol* (Ortho) (Acid. ricinolic. 0.75, propyl. paraoxibenz. 0.05, acid. boric. 3.0, acid. acet. cntr. 0.33, oxichinolin.sulf. 0.025, stannos. chlor. 0.015, glycerin. 10.0, gummi arabic. 2.0, tragac. 3.0, essent. 0.025, aq. dest. ad 100.0), and the corresponding Finnish *Obturol* (Orion): (Acid. ricinolic. 1.0, propyl. paraoxibenz. 0.05, acid. boric. 3.0, acid. acet. cntr. 0.33, oxichinolin. sulf. 0.025, stannos chlor. 0.015, glycerin. 10.0, essent. 0.025, tragac. et gummi arab. q.s., aq. dest. ad 100.0), and the English *Volpar* paste (BDH): (Phenyl mercur.

acet. 0.030, methyl parahydroxybenz. 0.055, Starch 2.275, sodium oleat. 1.165, cream base ad 100.0).

The vaginal suppositories examined were a Danish preparation *Anticon* (Medix) and the corresponding Finnish preparations ***Meracen*** (Orion), and ***Gynophen*** (Lääke) which all contain about 1.5 mg of phenyl-mercuri acetate per suppository.

We examined the sperms of 22 men, of whom 20 had children. The samples were 1—5 hours old, when the motility of the sperms still is good. According to Genell (1), the motility of the sperms is only slightly reduced after three hours 40 minutes. According to Varnek (2), the motility of the sperms is hardly reduced at all within 6 hours.

Method. — The contraceptives to be tested were suspended in saline for at least 10 mins. in a water-bath at +37° C before the examination. After this 0.03 cc of the sperm was sucked into a pipette of 0.1 cc; after a small bubble of air an equal quantity of the suspension of the contraceptive substance was sucked up. These were then blown on an object glass and quickly mixed with the point of the pipette (pipette test). After being

TABLE 1

EFFECT OF ANTICON, GYNOPHEN, AND MERACEN VAGINAL SUPPOSITORIES ON THE MOTILITY OF THE SPERMS

Sperm No.	Anticon				Gynophen				Meracen				Control	
	Pipette Test		Drop Test		Pipette Test		Drop Test		Pipette Test		Drop Test		Pipette and Drop Test	
	1′	2′	1′	2′	1′	2′	1′	2′	1′	2′	1′	2′	1′	2′
1	0		0		0		0		0		0		+++	+++
2	0		0		0		0		0		0		+++	+++
3	0		0		0		0		0		0		++	++
4	0		0		0		0		0		0		+++	+++
5	0		0		0		0		0		0		+++	+++
6	0		0		0		0		0		0		+++	+++
7	0		0		0		0		0		0		+++	+++
8	0		0		0		0		0		0		+++	+++
9	0		0		0		0		0		0		+++	+++
10	0		0		0		0		0		0		+++	+++
11	0		0		0		0		0		0		+++	+++
12	0		0		0		0		0		0		+++	+++
13	0		0		0		0		0		0		+++	+++
14	0		0		0		0		0		0		+++	+++
15	0		0		0		0		0		0		+++	+++

TABLE 2

EFFECT OF OBTUROL AND ORTHO-GYNOL ACID JELLIES AND OF VOLPAR PASTE ON THE MOTILITY OF SPERMS

Sperm. No.	Obturol				Ortho-Gynol						Volpar					Control		
	Pipette Test		Drop Test		Pipette Test				Drop Test		Pipette Test			Drop Test		Pipette and Drop Test		
	1′	2′	1′	2′	1′	2′	3′	4′	1′	2′	1′	2′	5′	1′	2′	1′	2′	5′
1	0		0		+	±	0		0		+++	+++	+	+++	+++	+++	+++	+++
2	±	0	0		0				0		+++	+++	+	+++	+++	+++	+++	+++
3	+	0	0								+	±	0	±	0	++	++	++
4	±	0	±	0	0				0		+++	+++	++	+++	+++	+++	+++	+++
5	0		0		0				0		+++	+++	+	+++	+++	+++	+++	+++
6	0		±		0				0		+++	+++	++	+++	+++	+++	+++	+++
7	0		±	0	0				0		+++	+++	+	+++	+++	+++	+++	+++
8	++	0			++	+	±	0			+++	+++	+++	+++	+++	+++	+++	+++
9	+	0			++	0					+++	+++	++	+++	+++	+++	+++	+++
10	0		0		0				±	0	+++	+++	++	+++	+++	+++	+++	+++
11	+	0	0		0				0		+++	+++	+++	+++	+++	+++	+++	+++
12	0		0		0				0		+++	+++	+++	+++	+++	+++	+++	+++
13	0		0		0				0		+++	+++	+++	+++	+++	+++	+++	+++
14	±	0	0		++	+	0		0		+++	+++	+++	+++	+++	+++	+++	+++
15					±	0			0		+++	+++	+++	+++	+++	+++	+++	+++

covered with the cover glass the slide was examined immediately with a microscope of low magnifying power to obtain a general impression, and then with a microscope of high magnifying power for details .In the first 15 samples the point of contact between the suspension of the contraceptive substance placed on the glass slide and covered with the cover glass and the drop containing sperms was also examined: by doing so, it was possible to study the properties of the substance affecting the motility of the sperms (drop test). In general at least two tests were carried out with each sample on the substance to be examined, and in the cases in which moving sperms were found after 1 min. several tests were performed.

The results of the pipette and the drop tests appear from the following tables, in which the different degrees of motility of the sperms are marked: +++ ++, +, and 0:

The mobility of the sperms was checked many times during each test. Sample No. 3 was poor; the motility of the sperms ceased during the test.

In addition to these, 7 samples were examined later, with Volpar paste used alongside of the other preparations. Both old and fresh preparations (the old preparation had been stored for more than a year at the chemist' and the fresh for 3 months were used. The results appear from Table 3.

TABLE 3

EFFECT OF GYNOPHEN VAGINAL SUPPOSITORY, OF ORTHO-GYNOL ACID JELLY, AND OF VOLPAR PASTE ON THE MOTILITY OF SPERMS

Sperm No.	Gyno-phen		Ortho-Gynol		Volpar						Control	
	Pipette Test		Pipette Test		Stale Pipette Test			Fresh Pipette Test			Pipette Test	
	1′	2′	1′	2′	1′	2′	5′	1′	2′	5′	1′	5′
16	0		0		++	+	0	++	+	0	+++	+++
17	0		+	0	+++	+++	+++	+++	+++	+++	+++	+++
18	0		0		+++	+++	++	+++	+++	+++	+++	+++
19	0		0		+++	+++	+++	+++	+++	+++	+++	+++
20	0		0		+++	+++	+++	+++	+++	+++	+++	+++
21	0		0		+++	+++	+++	+++	+++	+++	+++	+++
22	0		0		+++	+++	0	+++	+++	0	+++	+++

The motility of sample No. 16 was poor.

After 2 minutes it was not possible to establish any difference between the effect of long-stored and fresh contraceptives. Whereas with long-stored Volpar paste the motility of the sperms generally ceased after 5—10 mins., it ceased a little earlier when fresh Volpar was used. The difference was, however, so small as to be of no practical significance. For the sake of control the last test with sperm No. 22 was performed in a room at a temperature of +37° C. There was no essential difference in comparison to the tests made at room temperature. As a result of the sample drying up, the motility of the sperms ceased with Volpar after about 4 min.

It appeared that the spermicidal effect of the vaginal suppositories Anticon, Gynophen, and Meracen was extremely good *in vitro*. Not in a single case were moving sperms found after 1 min. Preparing this sample does not in fact take more than 10—20 seconds, and even then no motility was noticed in any of the samples. At the drop tests the motility ceased within a few seconds after coming into contact with the contraceptive suspension.

As to the acid jellies, at the pipette test motility was found in 7 samples after 1 min. when using Obturol, but after 2 mins. it had ceased in every case. At the drop test, living sperms were found in 3 samples after 1 min. At the corresponding tests with Ortho-Gynol, living sperms were found after 1 min. in 5 samples at the pipette test, after 2 mins. in 3 samples, and in one sample after 3 mins. Control tests were carried out with preparations kept for a week in a water-bath at +37°. (Sample-test No. 8). At the drop test only one sample showed moving sperms at the point of contact after 1 minute (12 cases).

On the other hand, the effect of Volpar paste proved to be doubtful. With the exception of samples Nos. 3 and 16, in which the motility of the sperms was poor, the motility at the pipette test was regularly almost

the same after 2 mins. and living sperms were found even after 5 mins. The same was found to be the case at the drop tests.

We also examined the melting of the vaginal suppositories in the vagina and the spreading of the contraceptive substance in it. In order to find out in which position in the vagina the contraceptive suppository has been placed, we examined its position immediately after the patient had introduced it into the vagina. In most cases the suppository was on the right lateral or front bottom. In 13 cases examined the complete melting of the suppository took place within 15—20 min.; after 5 mins. generally only about $^1/_3$ had melted. (The temperatures of all the patients were normal.)

The spreading of the contraceptive substance in the vagina was made clearly visible using Gynophen suppositories containing gentian violet (Lääke). In this manner the spreading of the colouring matter in the whole vagina was established within about 10 minutes.

SUMMARY

The writers examined *in vitro* the spermicidal effect of the vaginal suppositories Anticon, Gynophen, and Meracen, which appeared to be extremely good. The effect of the acid jellies Obturol and Ortho-Gynol was also rather good, though slower than the former, whereas the spermicidal effect of Volpar paste obtainable at the chemist's appears to be slow.

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FROM THE WIHURI RESEARCH INSTITUTE AND FROM THE DEPARTMENT OF PHARMACOLOGY, UNIVERSITY OF HELSINKI

EFFECT OF VARIOUS DRUGS IN EXPERIMENTAL PULMONARY OEDEMA

by

PENTTI I. HALONEN and JUHA HAKKILA

(Received for publication January 18, 1952.)

The classic theory of pulmonary oedema explained this syndrome as a result of an acute failure of the left ventricle. This view is no longer accepted by physiologists, whose opinion is that pulmonary oedema is due to a change in the permeability of the pulmonary capillaries. Physiologists have not, however, been able to elucidate the basic cause of this change. Neurogen factors, chemical toxins, and anoxia have been mentioned as causative agents. When the permeability of the capillaries is increased, a rise in the capillary pressure and haemodilution act as contributory factors in the genesis of pulmonary oedema.

Pulmonary oedema has been produced in experimental animals by several methods. Thus, ligation of the aorta (2, 14, 25, 27), or a lesion of the left or the right ventricle (7, 8) may produce pulmonary oedema. Similarly, an intravenous injection of saline after vagotomy (5, 6, 10, 15, 22, 28), or a rapid injection of saline into the carotid artery (19) also lead to pulmonary oedema. Further, this condition may be produced by administering adrenaline intravenously (4, 12, 23), ammonium chloride *per os* or by the intraperitoneal route (13), or with the aid of α-naphtyl thiourea (21) intraperitoneally. Some poisonous gases and hypertonic solutions given via the trachea also produce pulmonary oedema (3, 10, 16, 24, 29). Lastly, pulmonary oedema has also been produced by lesions of the central nervous system (5, 10, 11, 26).

As the mechanism of pulmonary oedema has not yet been finally elucidated, it seems obvious that the results of the treatment cannot always be optimal. Pulmonary oedema still shows quite high mortality figures. Because a number of new drugs have been marketed lately which are related or act in a similar way to substances known to have a favourable action on pulmonary oedema, it was deemed justified to study the effects of some of these drugs in experimental pulmonary oedema. Since the various types of experimental pulmonary oedema show great differences, several different methods of producing pulmonary oedema were used in this study.

MATERIAL AND METHODS

The experiments were made on guinea pigs and rats. Pulmonary oedema was induced in the guinea pigs by using adrenaline or ammonium chloride. Adrenaline was administered, in accordance with McKay & Pecka (20), intravenously, in a dose of 0.3 mg. per kg. Ammonium chloride was given, following the method of Koenig & Koenig (13), intraperitoneally, in a dose of 500 mg per kg. In rats, pulmonary oedema was produced by the aid of the method of MacKenzie & MacKenzie (21), by injecting α-naphtyl thiourea (ANTU) as an alcoholic-aqueous suspension intraperitoneally, in a dose of 250 mg per kg. When assessing the degrees of pulmonary oedema, attention was paid to the respiratory rate, to the appearance of bloody foam in the nostrils and mouth, and to the duration of life of the experimental animals. The lungs of the animals which had died or had been killed at a pre-determined time were examined, as to their general appearance and to the occurrence of foam in the trachea and in the pulmonary tissue. The lungs were weighed immediately and after drying. The dry weights are not included in the tables, as the wet weight alone appeared to give more valuable information. If the pleural cavity contained fluid, its volume was measured with the aid of a pipet. The presence of pulmonary oedema was also checked in histological preparations. The lungs to body ratio was determined by dividing the weight of the lungs in grams by that of the animal in grams and multiplying the results by 100.

The preventive action on experimental pulmonary oedema of

the following substances was studied: 1-methyl-4-m-oxyphenyl-piperidin-4-aethylketon-hydrochloride (Cliradon), 1-6-dimethyl-amino-4-4-diphenyl-heptanon-3-hydrochlorid (Algidon), α-ω-bis-trimethylammonium-hexan-dibromide (Vegolysen), ergotamine tartrate (Gynergen), and dimethoxymetil-furan-chromon (Khellin). The control material used comprised both untreated animals in which pulmonary oedema was produced and animals treated with morphine for pulmonary oedema. In preliminary experiments it was found that suitable doses were 7.5 mg of Cliradon, 10 mg of Algidon, 2 mg of Vegolysen, 0.5 mg of Gynergen, 15 mg of Khellin, and 15 mg of morphine as given intraperitoneally before the production of the experimental pulmonary oedema. 104 guinea pigs and 34 rats were used in the study.

PULMONARY OEDEMA PRODUCED WITH ADRENALINE

0.3 mg per kg of adrenaline given intravenously produces pulmonary oedema in guinea pigs with regularity, and the animals recover from it. The compounds under investigation were first tested by administering them before injecting adrenaline, and the animals were killed after 15-minutes. The degree of the oedema was assessed immediately. The results are shown in Table 1 and in Fig. 1.

It appears that Cliradon, Gynergen, and Khellin have a definite

TABLE 1

INFLUENCE OF VARIOUS DRUGS ON ADRENALINE PULMONARY OEDEMA IN GUINEA PIGS (ADRENALINE 0.3 MG/KG)

Drug	Number of Animals	Average Weight of Animals in Grams	Average Lung Weight in Grams	Average Lung-Body Ratio
Normal animals	8	480	2.98	0.62
Adrenaline (controls) ..	6	425	8.29	1.95
Morphine hcl (15 mg/kg)	5	580	5.13	0.88
Cliradon (7.5 mg/kg) ..	4	525	4.66	0.89
Algidon (10 mg/kg) ..	4	608	8.16	1.34
Vegolysen (2 mg/kg) ..	4	518	7.94	1.53
Gynergen (0.5 mg/kg)..	4	595	5.32	0.89
Khellin (15 mg/kg)....	5	479	5.36	1.12

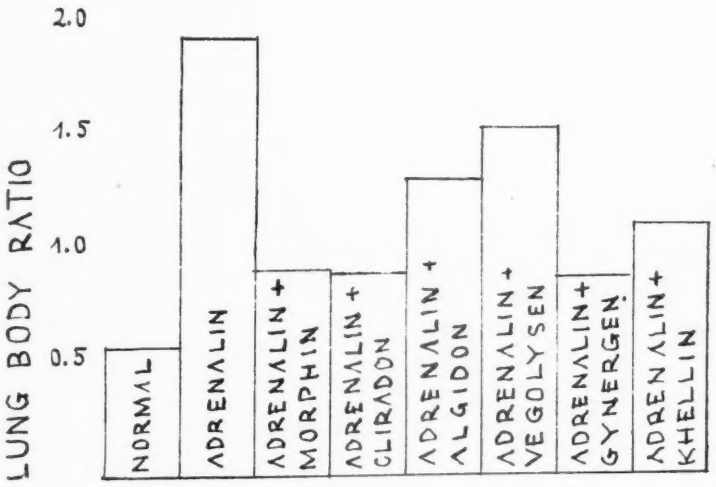

Fig. 1. — Different actions of drugs in adrenaline (0.3 mg/kg) produced pulmonary oedema.

preventive action on pulmonary oedema produced with the aid of 0.3 mg of adrenaline per kg, as assessed from the extent of the oedema. Their preventive action was about the same as that of morphine. Vegolysen and Algidon had also some preventive action, but this was definitely less marked than that of the three above-mentioned drugs.

Administered in larger doses, adrenaline produces a more severe pulmonary oedema. After a dose of 1 mg per kg, the resulting pulmonary oedema is severe enough to kill almost half of the experimental animals within 15 minutes. The drugs under investigation were tested also in this type of pulmonary oedema. The results are shown in Table 2 and in Fig. 2.

A preventive action on the more severe type of pulmonary oedema as produced by using adrenaline could also be observed in all of the drugs employed. The preventive action of Khellin was most pronounced, and that of Algidon seemed weakest.

These observations are in agreement with previous information. It is known that different narcotics, as e.g. morphine and chloralose, have a preventive action on pulmonary oedema as produced with adrenaline (18). Dihydroergotamine has a preventive action on

TABLE 2

INFLUENCE OF VARIOUS DRUGS ON ADRENALINE PULMONARY OEDEMA IN GUINEA PIGS (ADRENALINE 1 MG/KG)

Drug	Number of Animals	Dead under 15 Min. Number of Animals	Average Lung Weight in Grams	Average Lung-Body Ratio
Normal animals	8	—	2.98	0.62
Adrenaline (controls) ..	6	3	9.53	2.05
Morphine hcl (15 mg/kg)	4	—	4.76	0.93
Cliradon (7.5 mg/kg) ..	6	2	5.43	1.29
Algidon (10 mg/kg) ..	5	2	6.60	1.63
Vegolysen (2 mg/kg) ..	4	1	5.02	1.41
Gynergen (0.5 mg/kg)..	4	—	4.50	1.34
Khellin (15 mg/kg)....	9	3	4.11	0.91

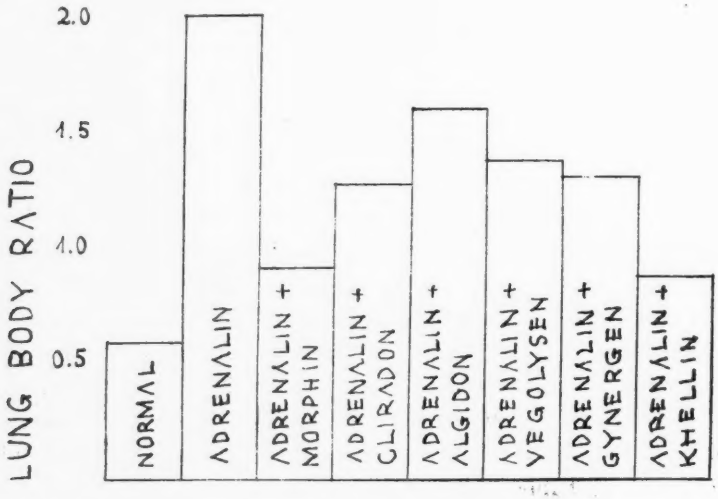

Fig. 2. — Different actions of drugs in adrenaline (1 mg/kg) produced pulmonary oedema.

pulmonary oedema ad produced in rabbits by adrenaline (9) and Gynergen a similar action in pulmonary oedema due to phosgen (24). It may be mentioned also in this connection that Anrep, Kenawy and Barsoum (1) mention that Khellin relieves attacks of cardiac asthma due to coronary thrombosis.

PULMONARY OEDEMA PRODUCED WITH AMMONIUM CHLORIDE

Ammonium chloride in a dose of 500 mg per kg given intraperitoneally produces pulmonary oedema regularly in guinea pigs, and this conditions always terminates in death within 30 min. The lungs show severe changes, haemorrhage, oedema, and congestion. For this reason, pulmonary oedema as produced by ammonium chloride is a less suitable form for experiments of this kind. A similar conclusion has been made also by Luisada. Nevertheless, the action of the above drugs, except for Khellin, was studied also in pulmonary oedema produced with the aid of ammonium chloride. The dosage and the route of administration were the same as in the adrenaline experiments described above. The results are shown in Table 3 and in Fig. 3.

TABLE 3

THE EFFECT OF VARIOUS DRUGS IN AMMONIUM CHLORIDE PULMONARY OEDEMA IN GUINEA PIGS

Drug	Number of Animals	Average Weight of Animals in Grams	Average Lung Weight in Grams	Average Lung-Body Ratio
Normal animals	8	480	2.98	0.62
NH_4 CL (controls)	5	501	5.36	1.07
Morphine hcl (15 mg/kg)	4	495	6.93	1.40
Cliradon (7.5 mg/kg) ..	4	509	4.73	0.93
Algidon (10 mg/kg) ..	4	553	5.81	1.05
Gynergen (0.5 mg/kg)..	4	485	4.87	1.00
Vegolysen (2 mg/kg) ..	5	506	4.55	0.90

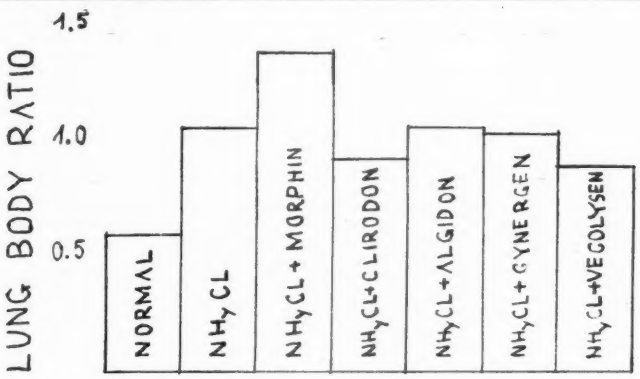

Fig. 3. — Ammonium chloride pulmonary oedema.

None of the drugs tested had a preventive action on pulmonary oedema as produced with ammonium chloride. Nor had morphine any preventive action. The duration of life of the treated animals and that of the controls showed no significant differences.

PULMONARY OEDEMA PRODUCED WITH α-NAPHTYL-THIOUREA (ANTU)

Pulmonary oedema as produced with the aid of ANTU in rats has been regarded as irregular, and therefore, it has not been looked upon as a suitable method for an assay (18). This result appeared to be confirmed in the first experiments of the present writers, when attempting to produce pulmonary oedema by using ANTU. This irregularity was evidently caused by the circumstance that it is difficult to produce a homogeneous aqueous suspension of ANTU. If ANTU is first wetted with alcohol and then suspended in water, a satisfactory suspension can be produced. An injection of 250 mg per kg body weight of such an aqueous suspension of ANTU resulted regularly in the formation of pulmonary oedema, terminating fatally within 10 to 15 hours. In addition to the pulmonary changes, the pleural cavity regularly contained exudate-like fluid. The same drugs were tested as in the adrenaline oedema. The results are shown in Table 4 and Fig. 4.

The pulmonary oedema as produced by ANTU was less pronounced than that produced by adrenaline in guinea pigs.

None of the drugs used had a preventive action on the degree

TABLE 4

THE EFFECT OF VARIOUS DRUGS ON PULMONARY OEDEMA CAUSED BY ANTU IN RATS

Drug	Number of Animals	Average Weight of Rats in Grams	Average Lung Weight in Grams	Average Lung-Body Ratio	The Amount of Fluid in the Pleural Cavity in cc
Normal animals	6	303	1.39	0.46	—
ANTU (controls)	5	255	1.94	0.76	6.1
Morphine hcl (15 mg/kg)	5	304	2.49	0.82	4.6
Cliradon (7.5 mg/kg) ..	4	273	2.28	0.84	3.2
Algidon (10 mg/kg) ..	4	221	2.52	1.14	4.3
Gynergen (0.5m g /kg)..	4	239	2.39	1.00	3.0
Vegolysen (2 mg/kg) ..	4	254	2.24	0.88	4.8
Khellin (10 mg/kg)....	4	213	1.77	0.83	5.0

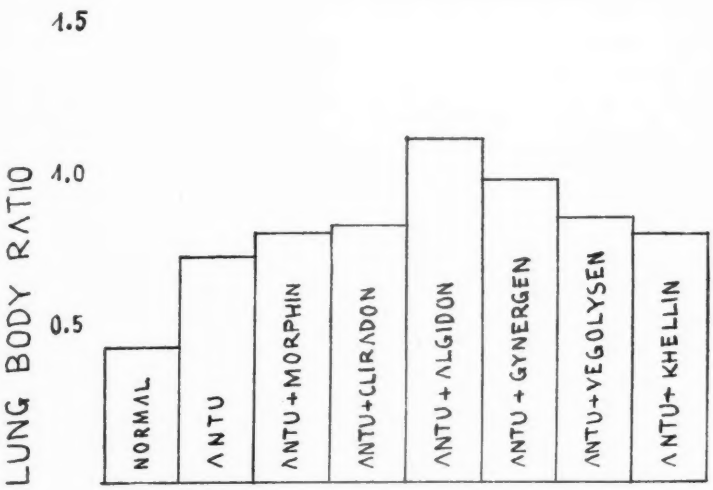

Fig. 4. — ANTU-produced pulmonary oedema.

of the pulmonary oedema, rather to the contrary. However, the amount of the fluid in the pleural cavity seemed to be reduced. The duration of the life was increased in comparison to the controls in all groups, by an average of 25 per cent.

SUMMARY

Experimental pulmonary oedema was produced in guinea pigs with the aid of adrenaline and ammonium chloride, and in rats by using *α*-naphtyl-thiourea (ANTU). Among these methods the use of adrenaline seems to be the most practical method when the action of drugs on experimental pulmonary oedema is being investigated. *α*-naphtyl thiourea produces pulmonary oedema in rat with regularity, contrary to earlier reports.

1-methyl-4-m-oxyphenyl-piperidin-4-aethyl-keton-hydrochloride (Cliradon), ergotamine tartrate (Gynergen), and dimethoxy-metil-furan-chromon (Khellin) have a preventive action on pulmonary oedema produced by adrenaline. Their preventive action is similar to that of morphine. *α-ω*-bis-trimethyl-ammonium-hexan-dibromide (Vegolysen) and 1-6-dimethyl-amino-4-4-diphenylheptanon-3-hydrochloride (Algidon) have also a preventive action, but this action is less pronounced than that of the above drugs.

These drugs were tested also on pulmonary oedema produced with ammonium chloride in guinea pigs and with α-naphtyl thiourea in rats; the results were not so definite as in the adrenaline oedema. This probably depends on the toxicity of these substances and on differences in the causative mechanism of pulmonary oedema.

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AUS DEM WIHURI'SCHEN FORSCHUNGSINSTITUT ZU HELSINKI

UEBER DIE LEUKOZYTENANZAHL IM PERIPHEREN UND IM HERZBLUT

von

PENTTI I. HALONEN und KAARLO E. J. KYLLÖNEN

(Der Schriftleitung zugegangen am 18 Jan. 1952)

Verhältnismässig geringe Aufmerksamkeit ist der eventuellen Verschiedenheit der Leukozytenmenge in den verschiedenen Teilen des Zirkulationsorganismus zugewandt worden. Law und Heston (1) bestimmten bei Ratten unter Verwendung der Nembutalnarkose die Anzahl der Leukozyten im Herz- und im Schwanzblut und stellten dabei beträchtliche Unterschiede fest (Schwanzblut 21510, Herzblut 3717/mm³). Quimby, et al. (3) betäubten die Ratten durch einen Schlag auf den Kopf und stellten im Schwanzblut durchschnittlich 23810 Leukozyten, im Herzblut dagegen nur 6425 Leukozyten pro mm³ fest. Die von ihnen erhaltenen Unterschiede konnten nicht durch Haemodilution bedingt sein, denn die Anzahl der Erythrozyten und das spezifische Gewicht des Blutes waren in den Blutproben beider Gruppen gleich. Nichols und Miller (2), welche bei ihren Versuchen leichten Aetherrausch oder Schlag auf den Kopf anwandten, konnten in der Leukozytenmenge im Herz- und Schwanzblut ihrer Ratten keine statistischen Unterschiede wahrnehmen. Smith, et al. (5) berichten, dass während Aethernarkose bei der Katze die Anzahl der Leukozyten sowohl im Herz- als auch im Ohrmuschelblut stieg, während sie bei Nembutalnarkose wiederum abnahm. Am deutlichsten waren die Veränderungen im Herzblut. Der Aether verursachte gleichzeitig Haemokonzentration, das Nembutal Haemodilution. Roofe et al. (4) haben festgestellt, dass dass Meerschweinchen in der Ohrmuskel mehr Erythrozyten und

Leukozyten hat als im Herzbut. Bei ihren Versuchen nahmen sie zuerst von der Ohrmuschel eine Blutprobe und danach in leichter Nembutalnarkose die Herzblutprobe, indem sie das Herz punktierten ohne den Thorax zu öffnen.

Da die Anzahl der Leukozyten sowohl im peripheren als auch im Herzblut beträchtliche theoretische Bedeutung hat, und da die früheren Resultate sehr widersprechend gewesen sind, haben wir es für angezeigt erachtet, diese Frage zu behandeln. Als Versuchstiere benutzten wir 25 Ratten, 8 Kaninchen und 2 Katzen. Die Proben des peripheren Blutes wurden bei den Ratten aus der Schwanzvene entnommen, bei den Kaninchen und den Katzen aus der Ohrmuschel. Die Herzblutproben wurden entweder durch Punktion des Herzens durch den Thorax hindurch oder durch Eröffnung der Brusthöhle und Punktion der rechten und linken Kammer des schlagenden Herzens genommen. Die Bestimmung der Leukozyten wurde in gewöhnlicher Weise vorgenommen, und zwar immer von der gleichen Person, welcher es unbekannt war, von wo die Blutprobe entnommen war. Nur solche Blutproben wurden berücksichtigt, bei welchen Koagulation überhaupt nicht und Veränderungen in der Haematokrite oder Veränderungen in der Anzahl der Erythrozyten nicht mehr als 10 % vorkamen. Ein Teil der Tiere wurde durch einen Schlag auf den Kopf betäubt, ein Teil bekam Aether-, Uretan- oder Chloralosenarkose unmittelbar vor Entnahme der Proben, bei einem Teil wurde längerwährende Narkose angewandt, während welcher mehrere Blutproben genommen wurden, bei den letzteren geschah die Atmung durch eine Trachealkanüle mit Hilfe einer Atmungspumpe. Die Resultate gehen aus der beigefügten Tabelle hervor.

Bei der Beurteilung der Ergebnisse ist grosse Vorsicht am Platze. Es ist ja bekannt, dass die Leukozyten in den verschieden grossen Blutgefässen sich in verschieneder Weise bewegen, indem sie sich in den dünneren Venen an die Gefässwand anlegen. In den dünnen Blutgefässen sind ausserdem Unterschiede auch in der Stromgeschwindigkeit der verschiedenen Leukozytenarten beobachtet worden (7). Ebensowenig kann man von der gleichmässigen Verteilung der Leukozyten im Blut des Herzens sicher sein.

Wenn man die obigen Tabellen Nr. 1 und 2 betrachtet, so fällt ein beträchtlicher Unterschied in der Anzahl der Leukozyten im Herz- und im peripheren Blut bei den durch Schlag auf den Kopf

TABELLE 1

ANZAHL DER LEUKOZYTEN IM SCHWANZ- UND IM HERZBLUT BEI DURCH SCHLAG AUF DEN KOPF BETÄUBTEN RATTEN. INTAKTER KREISLAUF

Ratte Nr.	Schwanzblut	Herzblut
1	12,500	4,500
2	17,300	4,500
3	31,700	11,100
4	19,800	5,000
5	20,100	9,500
6	17,500	18,000
7	20,000	4,700
8	16,400	8,300
9	16,100	14,100
10	18,100	6,100
Durchschnittlich	18,950	8,580

TABELLE 2

ANZAHL DER LEUKOZYTEN IM SCWANZBLUT SOWIE IM BLUT DER RECHTEN UND LINKEN HERZKAMMER BEI DURCH SCHLAG AUF DEN KOPF BETÄUBTEN RATTEN. HERZBLUTPROBEN DURCH ERÖFFNUNG DER BRUSTHÖHLE UND PUNKTION DER RECHTEN UND LINKEN KAMMER DES SCHLAGENDEN HERZENS ENTNOMMEN

Ratte Nr.	Schwanzblut	Blut der rechten Herzkammer	Blut der linken Herzkammer
1	12,500	2,400	1,500
2	23,800	8,700	6,100
3	19,100	8,300	4,700
4	16,700	10,100	4,500
5	16,100	5,300	4,300
6	33,500	9,300	3,900
7	16,700	4,400	2,300
8	21,200	11,650	5,850
9	20,100	5,550	4,350
10	16,800	4,600	3,150
Durchschnittlich	19,650	7,030	4,065

betäubten Versuchstieren ins Auge. In diesen Fällen besteht auch ein deutlicher Unterschied in der Leukozytenanzahl des Blutes der rechten und der linken Herzkammer. Die Unterschiede in der Anzahl der Leukozyten zwischen dem peripheren und dem Herzblut sind ähnlich wie die von Law und Heston (1) sowie Quimby und Mitarbeiter (3) beobachteten. Die Unterschiede sind so gross, dass man sie nicht mehr durch technische Umstände erklären kann. Ähnliche Differenzen liessen sich auch bei zwei durch Schlag auf

TABELLE 3

DURCHSCHNITTLICHE ANZAHL DER LEUKOZYTEN BEI DEN VERSUCHSTIEREN IM PERIPHEREN BLUT SOWIE IM BLUT DER RECHTEN UND LINKEN HERZKAMMER IN NARKOSE

Versuchstier und Anzahl	Narkose	Probe genommen	Durchschnittl. Anzahl d. Leukozyten			Bemerkungen
			Periph.	Rechte Kammer	Linke Kammer	
Kaninchen 2	Aether	Unmittelbar	6,250	5,500	4,350	
Katzen 2	»	»	18,250	13,575	12,625	
Kaninchen 1	Uretan	»	10,000	7,300	6,800	
Ratten 5	Chloralose	»	15,710	9,510	8,390	
Ratten 5	»	nach 2 Stunden	10,320	5,540	5,050	Brusthöhle geöffnet. Atmung mit Blasebalg durch Tracheakanüle.
Kaninchen 3	»	unmittelbar	14,150	11,750	10,200	
Kaninchen 3	»	nach 2 Stunden	13,080	6,960	7,400	

den Kopf betäubten Kaninchen fesstellen, in deren Ohrblut durchschnittlich 10,450, im Blut der rechten Herzkammer 2,250 und im Blut der linken Herzkammer 1,250 Leukozyten vorkamen. In den während kurzem Aetherrausch genommenen Blutproben (Tabelle 3) von zwei Katzen und zwei Kaninchen sowie in Uretannarkose von einem Kaninchen waren die Unterschiede der Leukozytenanzahl im peripheren und im Herzblut viel kleiner. Bei Chloralosenarkose waren unmittelbar nach Beginn der Narkose kleinere und nach zweistündiger Dauer der Narkose beträchtlich grössere Unterschiede zwischen dem peripheren und dem Herzblut zu beobachten (Tabelle 3). Die Autoren können nicht beurteilen, in welchem Masse die Resultate von der angewandten künstlichen Atmung und der Eröffnung der Brusthöhle abhängig sind.

Es besteht die Möglichkeit, dass der Unterschied zwischen den früheren Untersuchungen und auch die Verschiedenheit unserer eigenen Beobachtungen in Bezug auf die durch kurze Narkose oder durch Schlag auf den Kopf betäubten Tiere auf die Schockwirkung zurückzuführen ist. Schon Webb (8) hat gezeigt, dass im anaphylaktischen Schock die Anzahl der Leukozyten im peripheren Blut rasch abnimmt, und grosse Unterschiede in der Leukozytenmenge des Blutes der rechten und der linken Herzkammer bestehen, was zunächst durch das Verbleiben der Leukozyten in den Lungen bedingt ist. Erwähnenswert ist, dass auch Studer (6) bei der Untersuchung der Anzahl der Leukozyten im peripheren und im Herz-

blut bei der Ratte nach Betäubung bedeutende Unterschiede hierin feststellte. Bei intakten Ratten waren die Differenzen bedeutend kleiner.

ZUSAMMENFASSUNG

Die Autoren haben die Anzahl der Leukozyten im peripheren Blut sowie im Blut der rechten und der linken Herzkammer bei Ratten, Kaninchen und Katzen untersucht. Ein Teil der Tiere wurde durch einen Schlag auf den Kopf betäubt, ein Teil bekam Aetherrausch, oder es wurde kürzer- oder längerwährende Chloralosenarkose angewandt. Beträchtliche Unterschiede in der Anzahl der Leukozyten im periferischen Blut und im Herzblut waren der durch Schlag auf den Kopf betäubten Tiere festzustellen, wobei kleinste Werte im Blut der linken Herzkammer konstatiert wurden. In den im Aetherrausch entnommenen Blutproben wurden bedeutend kleinere Unterschiede beobachtet. Bei zwei Stunden langer Chloralosenarkose, wobei die Brüsthöhle eröffnet war und Druckatmung zu Hilfe genommen wurde, liessen sich beträchtliche Unterschiede in der Anzahl der Leukozyten des peripheren und des Herzbluts wahrnehmen.

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FROM THE DEPARTMENT OF ANATOMY, UNIVERSITY OF HELSINKI

CIRCULATING BAND NEUTROPHIL LEUCOCYTES OF THE RAT

EFFECT OF ESTROGEN

by

KALEVI KETTUNEN

(Received for publication January 22, 1952)

In the animal experiments reported in a previous paper (3) it was noticed that estrogen decreases the number of the red blood cells and hemoglobin concentration in the circulating blood. There is a simultaneous increase in the number of reticulocytes. The object of the present paper is to show the increasing effect of estrogen on the circulating bandform neutrophils.

EXPERIMENTAL MATERIALS AND METHODS

Adult female albino rats weighing 120—210 grams were used in these studies. The rats were fed on standard laboratory diet and tap water *ad libitum*. Total and differential leucocyte determinations were performed once a week by standard procedures on the blood obtained from the tails of the rats. The differential leucocyte values were computed after counting two hundred white cells on blood smears stained with May-Grünwald solution. All the counts were made in the afternoon in the post-absorptive state. The ovaries were removed through mid-ventral incisions. Ether was used for anesthetic purposes.

The leucocyte picture was studied in five different groups of rats as follows:

Group a. — Ten normal intact rats in our colony were elected at random for the determinations of leucocyte composition. These were considered the control group for this study.

Group b. — Eight rats comprising this group were given subcutaneous injections of estradiol benzoate every other day at a dose level of 1.5 mg per injection.

Group c. — The procedures in the eight rats comprising this series were exactly the same as the above group with the exception that the hormone dose was 15 μg per injection.

Group d. — In this series of ten rats leucocyte counts were made once before and once a week after the removal of the ovaries during the subsequent 6 weeks of the study.

RESULTS

The results were statistically evaluated. As to the variables under investigation it may be supposed that their rate of growth depends on the value already reached by them. Thus it may also be supposed that the so-called logarithmico-normal distribution can well approximate the distribution of the observation values within the groups. In fact, these distributions are somewhat skew, so these approximations can be well defended. For the calculations all the observation values were transformed into their logarithms. The averages below were also calculated from the logarithms, and thus they are approximate values of the geometrical means, and also approximate values of the »most probable value» or mode.

As a first approximation in describing the general course of the experiments we calculated the linear regressions with respect to time. The main results of the calculation are given in Table 1. The table shows that the observed regressions are insignificant ($P > 0.1$). This may be due to the fact that the applicable regression curve

TABLE 1.

LINEAR REGRESSIONS WITH RESPECT TO TIME AND THE TESTS ON THEIR RELIABILITY

Group	Trend of Regression	Residual Sum of Squares Compared with Total*	Correlation Coefficient	p**
a. Controls	±	1.0	0	>0.1
b. E.b. dose 1.5 mg	Increasing	0.9	+0.3	>0.1
c. E.b. dose 15 μg	Increasing	0.8	+0.5	>0.1
d. Ovariectomized	Decreasing	0.9	—0.3	>0.1

E.b. = Estradiol benzoate.

* This number shows what part of the total variation of the observations remains »unexplained», *i.e.*, what part is not at least due to a linear trend.

** P is the probability, that the observed regression is due to chance alone.

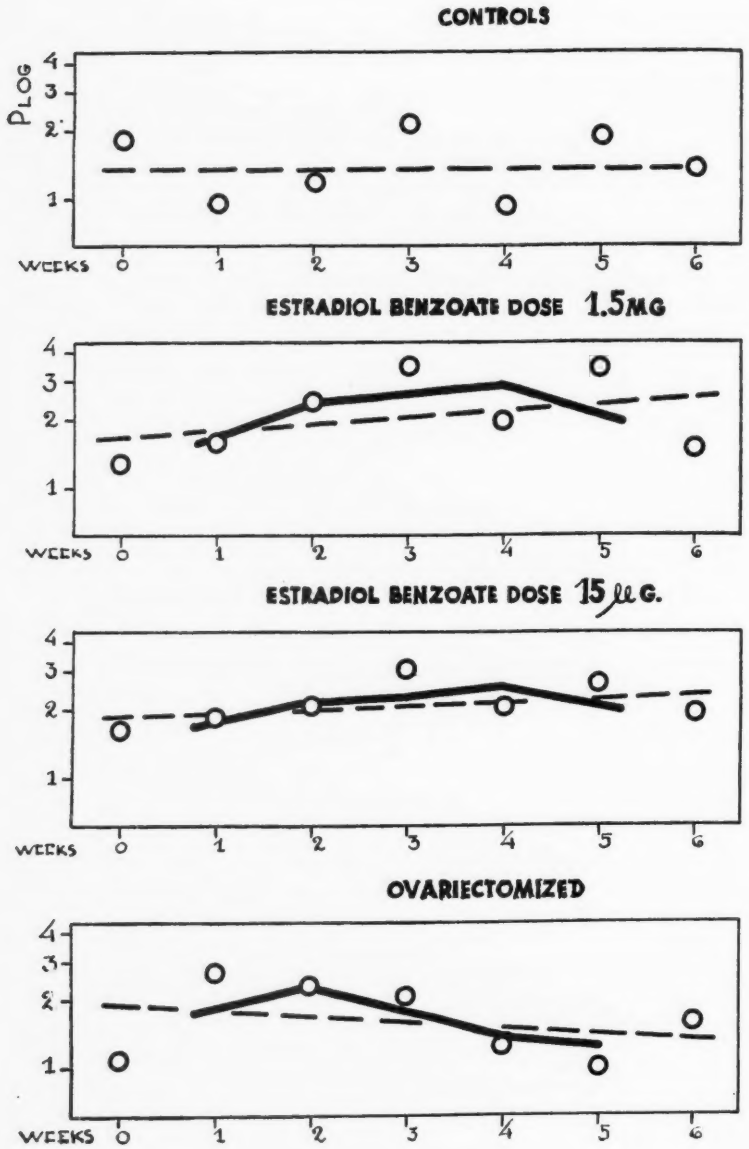

Fig. 1. — Effect of estrogen administration and ovariectomy on the circulating band neutrophil leucocytes of rats.

is not a straight line, and the relatively great variation in the material also weakens the description possibilities. A more detailed representation could be considered either with some polynomial curves or, *e.g.*, with moving averages. We chose the latter and calculated the moving averages for the weeks 1 to 5.

In Fig. 1, the scale of which is logarithmic, the curves obtained as the results of the calculations are presented. The small circles show the location of the geometrical means $G_i\ (y)$:

$$\log G_i\ (y) = \Sigma\ \log y/n_i,$$

y = individual observations
n = number of observations
i refers to observation week, $i = 0,1, \ldots,6$

The broken lines represent the regression lines Y:

$Y_i = a + bi$, a and b are the regression coefficients.

The moving averages $A_i\ (y)$,

$$\log A_i\ (y) = \frac{\sum_{t=i-1}^{i+1} \log G_t\ (y)}{3}$$

are connected with each other by straight lines, which are given as thick solid lines.

A visual examination of the moving averages gives rise to the supposition that they represent at least in some cases changes of a systematic nature. As the considerable variation of the material does not allow the performance of very complicated calculations we have given some suppositions of the general development shown by these means and tested these suppositions in the following simple way: —

Estradiol Benzoate Dose 1.5 mg per Injection. — It is supposed that the band cell percentage is during the weeks from 2 to 5 on the average essentially above the linear trend and at the other times essentially below it. By counting all the individual observations of these weeks we get the following contingency table:

Number of Observations	Week		Total
	2—5	0,1,6	
Above the trend	24	8	32
Below the trend	7	14	21
Total	31	22	53

By calculating the quantity χ^2 for the four-fold table in the usual way we get $\chi^2 = 9.1$. From the table proper we see that the probability (P), which shows that the value is due to chance alone, is $P < 0.01$. Thus we can, from a practical point of view, consider the supposition to be right.

Estradiol Benzoate Dose 15 μg per Injection. — The supposition as above. The following contingency table is obtained:

Number of Observations	Week		Total
	2—5	0,1,6	
Above the trend	18	12	30
Below the trend	14	11	25
Total	32	23	25

$\chi^2 = 0.8 \qquad P > 0.1.$

In this case our supposition is not verified.

Ovariectomized Rats. — It is supposed that the band cell percentage is during the weeks from 1 to 3 on the average essentially above the linear trend and during the weeks from 4 to 5 below it. The contingency table and the results are as follows:

Number of Observations	Week		Total
	1—3	4—5	
Above the trend	25	9	34
Below the trend	5	8	13
Total	30	17	47

$\chi^2 = 5.0. \qquad P < 0.05.$

We can approve the supposition as satisfactorily verified.

Thus the moving averages give, in general, a good picture of the chief development of the observations. In the above cases, in which a verified result was obtained, the result does not naturally verify anything of the individual values of the moving averages, but instead of the general development, the form of which is best observed in the figures.

DISCUSSION

The nuclear lobulation of the neutrophil leucocytes gives an indication of the age of the cell. If there is a sudden stimulus to the bone marrow to produce granulocytes, bandform cells increase rapidly in the blood. The number and the proportion of these young cells depends upon the nature of the stimulus and upon the reaction of the individuals.

A »shift to the left», an increase of the bandform cells at the expense of those with more lobes, occurs irrespective of leucopenia or leucocytosis. It is found in all infections, in toxemias and also after hemorrhage. Artifical fever, ultra-violet rays, vibratory massage and the induction current are said to produce a shift to the left (2). A right shift is found in pernicious anemia, in some cases of anemia in pregnancy and in vitamin deficiency diseases.

The present study shows that exogenous estrogen has an increasing effect on the circulating band neutrophil leucocytes. The results also indicate that the amount of the shift is proportional to the size of the dose. Dalton and Selye (1) pointed out in the rat that the eosinophils first decreased in number and then increased after various alarming stimuli. Selye (4) later included this pattern of response in his concept of the general adaptation syndrome. On the basis of this investigation there is evidence that with a continued exposure to estrogenic hormone administration an individual may acquire some power of resistance to its action.

If there is a sudden stimulus to the bone marrow to produce granulocytes, as in the case of ovariectomy in the present study, young cells appear rapidly in the blood. The later decrease in the number of bandform neutrophils after ovariectomy evidences the effect of internal secretion.

SUMMARY

Estradiol benzoate in a dose of 1.5 mg every other day for six weeks caused a significant increase in the circulating band neutrophil leucocytes of female rats. An increase caused by a dosage of 15 μg per injection was insignificant.

Ovariectomy caused an increase during the first week, after that a decrease.

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FROM THE DEPARTMENT OF SEROLOGY AND BACTERIOLOGY, UNIVERSITY OF HELSINKI

SEROLOGICAL STUDIES IN RHEUMATOID ARTHRITIS

II

ABSORPTION OF THE STREPTOCOCCAL AGGLUTINATING FACTOR FROM SERA OF PATIENTS WITH RHEUMATOID ARTHRITIS BY *Streptococcus haemolyticus* AND *Staphylococcus aureus*[1])

by

NILS OKER-BLOM

(Received for publication September 31, 1951)

Since our first paper (3) on this subject was published, the study of rheumatoid arthritis has entered a new phase concerned with the effects which follow the administration of the adrenocorticotrophic hormone (ACTH) or the hormone of the adrenal cortex (Cortisone) to patients with this disease. The cause of the disease (7) and the relation of the adrenal cortex to it (2,8) remain obscure.

Among theories proposed to account for this disease was the view that streptococci were responsible. The chief evidence for this concept was the agglutination of living hemolytic streptococci by serum from patients with rheumatoid arthritis (reviewed in the previous paper (3)). In other studies of the author (3, 4), however, some doubt has been expressed concerning the specifity of this agglutination reaction. One reason for this has been that some strains of *Staphylococcus aureus* are also agglutinated by serum from these patients in nearly the same degree as the hemolytic streptococcus. It has been suggested that the cause of this staphylo-

[1] An abbreviated report on the results presented in this and an earlier paper (3) was read before the Second Meeting of Scandinavian Rheumatologists, Stockholm, September 17—18, 1948.

coccal agglutination may be one or more of the following: (1) *Streptococcus haemolyticus* and *Staphylococcus aureus* have some antigen in common, (2) the etiology of rheumatoid arthritis is complex and serum from patients with the disease contains agglutinins against both bacteria, (3) the agglutination of these and certain other bacteria such as non-encapsulated pneumococci and enterococci are caused by some non-specific serologic factor or factors.

The experiments presented in this and subsequent papers were performed with a view to determine which of the above possibilities could be verified. This paper is concerned with the question of whether the serological capacity to agglutinate streptococci and staphylococci is attributable to a common antigen present in both organisms.

METHODS

Bacterial Strains. — *Streptococcus haemolyticus*, strain SF130, previously used for routine agglutination experiments, and *Staphylococcus aureus*, strain 1240, obtained from pus from a mammary gland fistula, were used. The latter organism was mannitol and coagulase positive.

Antigens. — The bacteria were cultivated in a one per cent Difco proteose peptone broth for 12—14 hours. After centrifugation at 2500 rpm. for 20 minutes the bacteria were resuspended in a small amount of saline and killed by keeping the suspension at 56°C for one hour. For immunization and titrations the suspension was diluted to contain about 300 millions of organisms per ml.

Titration Method. — Titrations were performed as previously described (3) with the exception that heated antigens prepared as described above were used instead of living organisms. The agglutinatination of living streptococci and streptococci killed at 56°C should be in all respects comparable (1).

Immune Serum. — Three times a week white rabbits weighing about 2000 grams each were injected intravenously with the antigen, beginning with a dose of 0.2 ml which was gradually increased to 2.0 ml. Two rabbits were immunized with streptococcal antigen, two with staphylococcal antigen and two with a mixture of both antigens in equal parts. Before the immunization and at intervals of one week during the immunization, samples of blood

were taken by heart puncture and the agglutination titer of each specimen was determined. Within four weeks satisfactory titers were obtained.

Human Serum. — Specimens of serum from ten patients with typical rheumatoid arthritis were used. Each of these sera had the capacity to agglutinate both streptococci and staphylococci in comparatively high titer.

Absorption Method. — Each serum was diluted 1: 10. Equal parts (two ml) of this diluted serum and of concentrated antigen were mixed (the antigen was concentrated by centrifuging the antigen and collecting the sediment). The mixture of antigen and serum was vigorously shaken and kept for two hours in a water bath at 37°C. After centrifugation at 2500 r.p.m. for 20 minutes the supernatant was removed and used as absorbed serum in agglutination tests.

RESULTS

Table 1 shows the final streptococcal and staphylococcal agglutination titers obtained with serum from each of three rabbits

TABLE 1

STREPTOCOCCAL AND STAPHYLOCOCCAL AGGLUTINATION TITERS OF SPECIFIC IMMUNE SERA BEFORE AND AFTER ABSORBTION WITH HOMOLOGOUS AND HETEROLOGOUS ANTIGEN

Rabbit	Immunized with	Strepto-coccal Aggl.Titer	Staphylo coccal Aggl.Titer	After Absorbtion with Streptococcal Antigen		After Absorbtion with Staphylococcal Antigen	
				Strept. Aggl. Titer	Staph. Aggl. Titer	Strept. Aggl. Titer	Staph. Aggl. Titer
789	strept.	2560	—	640	—	2560	—
790	strept. staph.	640	320	—	320	640	—
796	staph.	—	320	—	1280	—	—

following immunization against streptococcal antigen, staphylococcal antigen and a mixture of both antigens respectively. The corresponding titers of the same sera after absorption with homologous or heterologous antigen are also shown. It can be seen that the repeated injection of rabbits with heatkilled streptococci or staphylococci apparently gave rise to specific agglutinating antibodies, which was the case also if a mixed streptococcal and staph-

ylococcal antigen was used for the immunization. Moreover, from the results of cross absorption tests, there did not appear to be any obvious relation between these two different bacterial antigens when this method of testing was used, for absorbtion always resulted in removal of the homologous agglutinin only, even when the rabbits were immunized with the mixed antigen.

Thus, if identical antigens are not present in streptococci and staphylococci, and the agglutination of these bacteria by sera from patients with rheumatoid arthritis can be attributed to specific antibacterial antibodies, absorption of these human sera with bacterial antigens should be similar to those obtained with the specific immune rabbit sera. From the results presented in Table 2,

TABLE 2

STREPTOCOCCAL AND STAPHYLOCOCCAL AGGLUTINATION TITERS OF SERA OF PATIENTS WITH RHEUMATOID ARTHRITIS BEFORE AND AFTER ABSORPTION WITH STREPTOCOCCI AND STAPHYLOCOCCI

Serum	Streptococcal Aggl. Titer	Staphylococcal Aggl. Titer	After Absorption with Streptococcal Antigen		After Absorption with Staphylococcal Antigen	
			Strept. Aggl. Titer	Staph. Aggl. Titer	Strept. Aggl. Titer	Staph. Aggl. Titer
1014	320	320	—	160	—	—
1036	640	640	—	160	—	—
1230	640	160	—	160	—	—
1237	320	160	—	160	—	—
1238	640	320	—	640	—	—
1262	640	160	—	160	—	—
1289	640	320	—	320	—	—
1299	160	160	—	80	—	—
1300	160	320	—	320	—	—
1326	320	80	—	160	—	—

where the human sera were absorbed with streptococci and staphylococci in the same manner as the immune sera, it is clear that this is not the case.

The staphylococcal antigen seems to remove both homologous and heterologous »agglutinins», whereas the streptococcal antigen removes only the homologous »agglutinins». Thus, the sera of patients with rheumatoid arthritis seem to react quite differently

following absorbtion with streptococci and staphylococci than do specific rabbit immune sera.

Finally it is noteworthy that the agglutination tests are sometimes difficult to interpret either because the reaction may not be clear or because auto-agglutination may occur.

SUMMARY AND CONCLUSIONS

Experiments were carried out to determine whether the agglutination of both streptococci and staphylococci by sera from patients with rheumatoid arthritis could be ascribed to similar or identical antigens in the two bacteria. The injection of heat-killed streptococci or staphylococci into rabbits induced the development of specific agglutinins which were specifically absorbed only by the homologous antigen. If the rabbits were injected with a mixed streptococcal and staphylococcal antigen, specific agglutinins, which could be absorbed only by the homologous antigen, similarly developed. In tests with sera from patients with rheumatoid arthritis which agglutinated both streptococci and staphylococci heat-killed staphylococci absorbed both homologous and heterologous »agglutinins». With streptococci only homologous »agglutinins» were absorbed. The cause of these absorption results has not been explained, but on the basis of these findings and the evidence presented in the accompanying papers (5, 6), it seems most likely that the agglutination of streptococci and staphylococci by the patients' sera is caused by some non-specific factor or factors in the serum rather than by specific agglutinins.

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FROM THE DEPARTMENT OF SEROLOGY AND BACTERIOLOGY, UNIVERSITY OF HELSINKI

SEROLOGICAL STUDIES IN RHEUMATOID ARTHRITIS

III

COMPARISON BETWEEN THE AGGLUTINATION OF *Streptococcus haemolyticus* AND *Staphylococcus aureus* AND ANTISTREPTOLYSIN AND ANTISTAPHYLOLYSIN TITERS IN SERA OF PATIENTS WITH RHEUMATOID ARTHRITIS

by

NILS OKER-BLOM and OLOF WIDHOLM

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In previous papers (2, 3, 4) an attempt has been made to elucidate the nature of the agglutination of both streptococci and staphylococci by sera of patients with rheumatoid arthritis, and to determine the relation of these reactions to the etiology of rheumatoid arthritis. According to one of the previous papers (4) an antigenic relationship between the two bacteria in question could not be demonstrated, although the experiments did not completely exclude such a possibility. It seems doubtful, however, that sucha relationship could account for the agglutination of both streptococci and staphylococci that has so often been observed with sera from patients with rheumatoid arthritis. If the agglutination is specific and the assumption of identical antigenes can be disregarded, there remains the possibility that two different agglutinins may exist in the sera. If this were true, some kind of parallelism between the occurrence of agglutination and other antibodies might be expected. This paper presents the results of comparative studies of the agglutination, antistreptolysin, and antistaphylolysin reactions respectively.

MATERIAL AND METHODS

Sera. — Specimens of serum from patients with rheumatoid arthritis, and as control specimens, sera from children five months to ten years old wered used. Titrations of the streptococcal and staphylococcal agglutination with »normal» adult sera have been performed before (2), but not with sera from children. It seemed of interest to determine the frequency of positive streptococcal and staphylococcal agglutination in relation to the antistreptolysin and antistaphylolysin titers in children who are frequently infected by streptococci and staphylococci.

All the sera were inactivated at 56° C for half an hour and, if not used immediately, they were stored at —8 °C to —15 °C.

Agglutination Reactions. — For the streptococcal agglutination a living culture of the strain SF 130 was employed. The staphylococci previously used showed very different agglutinability. Therefore a number of strains were tested and the strain which showed the strongest agglutination in a test serum was selected. The staphylococci were also used as a living culture. The titrations were performed according to a method described previously (2). In this study a serum from a patient with rheumatoid arthritis which gave a strongly positive streptococcal and staphylococcal agglutination reaction was used as a control for the agglutinability of the organisms. The cultures were tested every day for purity and autoagglutination.

Antistreptolysin and Antistaphylolysin Titers. — These were both determined according to methods previously employed (5, 8).

RESULTS

Table 1 shows the agglutinability and autoagglutination on different days of the staphylococcus strains in the test serum compared to the streptococcal agglutination (Table 1).

The different strains of staphylococci differ widely in agglutinability in the same serum and on different days, and some of them show auto-agglutination. Some of the strains are old laboratory strains whereas others are recently isolated, but there does not seem to be any greater difference in agglutinability and in frequency

TABLE 1

AGGLUTINABILITY AND OCCURRENCE OF AUTOAGGLUTINATION AMONG SOME LABORATORY STRAINS AND SOME FRESHLY ISOLATED STRAINS OF *Staphylococcus aureus* AND OF ONE STRAIN OF *Streptococcus haemolyticus*

»Laboratory Strains»	Titers		Freshly Isolated Strains	Titers	
	May 12th	May 16th		May 12th	May 16th
Streptococcus SF 130	640	640			
Staphylococcus			staphylococcus		
1240	80	[1]	31974	160	160
1236	40	80	35	160	160
1165	160	80	21974	40	80
1253	80	[1]	209	[1]	[1]
1243	40	80	165	[1]	—
3707	80	80	2	40	80
11/111	320	320	1(2)	40	40
Orion	80	80	1	[1]	[1]
Plasma	160	160	10883	160	160

[1] auto-agglutination.

of auto-agglutination among recently isolated strains than among older ones. The factors most affecting the reaction seem to be the type of strain and especially the condition and age of the culture. Using the same strain and the same test serum the agglutination may be clear and strong with one culture and weak with another, and sometimes autoagglutination may occur even if the culture is prepared every time in exactly the same manner. The results obtained with the streptococcus strain used were, on the whole, more reliable but even with this strain auto-agglutination was quite common.

Although there were these difficulties with the agglutination reactions, the results obtained after several titrations seem to be fairly reliable. They are shown in Table 2.

The antistreptolysin titers in the »normal» children were often increased (41 per cent), which suggests a subclinical streptococcal infection, but these children did not show any positive streptococcal agglutination. The number of elevated antistaphylolysin titers was low and the children did not show any positive staphylococcal agglutination. Many of the patients have been followed for

TABLE 2

DISTRIBUTION OF POSITIVE STREPTOCOCCAL AND STAPHYLOCOCCAL AGGLUTINATION TESTS AND INCREASED ANTISTREPTOLYSIN AND ANTISTAPHYLOLYSIN TITERS AMONG THE CONTROL GROUP AND IN PATIENTS WITH RHEUMATOID ARTHRITIS

	Number Tested	Per Cent AST $\geq$ 200	Per Cent Strepto-coccal Aggl. $\geq$ 1:20	Per Cent Astaph $\geq$ 2	Per Cent Staphylo-coccal Aggl. $\geq$ 1:20
Control group.....	86	41	—	7	—
Rheumatoid arthritis	48	23	50	23	40

several weeks and their sera have been tested several times. In most cases the antistreptolysin and antistaphylolysin values do not change very much during the observation period. In some cases however there seems to be a rise in titer which later either drops or remains elevated, the latter speaking in favor of a specific infection. The values of the agglutination tests in the same patient at different times, however, have varied somewhat, being sometimes positive ($\geq$ 1: 20) and sometimes negative ($<$ 1: 20). The percentage of elevated antistaphylolysin titers in patients with

TABLE 3

FREQUENCY OF SIMULTANEOUSLY OCCURRING POSITIVE OR NEGATIVE STREPTOCOCCAL AND STAPHYLOCOCCAL AGGLUTINATION TITERS AND INCREASED SNTISTREPTOLYSIN AND ANTISTAPHYLOLYSIN TITERS, IN SERA OF PATIENTS WITH RHEUMATOID ARTHRITIS

	Number	AST $\geq$ 200	Astaph $\geq$ 2	Strepto-coccal Aggl. Positive	Staphylo-coccal Aggl. Positive
Streptococcal aggl. positive	24	4	4		12
Streptococcal aggl negative	24	10	7		7
	48	14	11		19
Staphylococcal aggl. positive	19	4	6	12	
Staphylococcal aggl. negative	29	10	5	12	
	48	14	11	24	

rheumatoid arthritis is far below that found by Westergren (7), but higher than the percentage of increased titers in the children reported here or in »normal» adult persons (Widholm 8). The main point of the investigation was, however, to find out whether there was any parallelism between the antistreptolysin and antistaphylolysin titers and positive streptococcal and staphylococcal agglutination tests respectively. The data of this comparison are presented in Table 3.

Thus there does not seem to be any correlation between increased antistreptolysin titers and positive streptococcal agglutination tests or between increased antistaphylolysin titers and positive staphylococcal agglutination tests. The parallelism between the staphylococcal and streptococcal agglutination tests is also less pronounced than in earlier experiments.

CONCLUSION

It is a well known fact that the antistreptolysin titer does not very often increase in rheumatoid arthritis (1), which is somewhat surprising consedering the positive streptococcal agglutination. If the cause of the streptococcal, agglutination is a streptococcal infection, one would expect occurrence of other streptococcal antibodies and thus also antistreptolysin, and one would also expect a parallelism between these two reactions. This is not the case. The same argument should be true also regarding the antistaphylolysin titer and staphylococcal agglutination, but also in this case it is impossible to find any correlation between the reactions.

Considering the above-mentioned facts and also the peculiar reaction of the sera to specific adsorption, compared with specific immune sera (4), and the different behavior of these sera and specific immune ones after treatment with bentonite (6), it seems most probable that this agglutination is caused by some non-specific factor or factors in the sera.

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FROM THE DEPARTMENT OF SEROLOGY AND BACTERIOLOGY, UNIVERSITY OF HELSINKI

SEROLOGICAL STUDIES IN RHEUMATOID ARTHRITIS

IV

ABSORPTION WITH BENTONITE OF THE STREPTOCOCCAL AGGLUTINATING FACTOR FROM SERA OF PATIENTS WITH RHEUMATOID ARTHRITIS[1]

by

NILS OKER-BLOM

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In the previous papers of this series the specificity of the agglutination of living hemolytic streptococci by sera of patients with rheumatoid arthritis was doubted, and it was suggested that the cause of this agglutination may be some non-specific factor or factors of the sera (3, 4, 5, 6).

In another connection bentonite was introduced in serological work for removing non-specific serum fractions, especially lipids and lipoproteins (2, 7). Bentonite has previously been used by Hansen (1) for removal of antiproteolytic serum fractions, *i.e.* lipids, for the purification of diphtheria antitoxin. Chemical and electrophoretic analyses have shown that bentonite added in suitable amounts to a dilute serum removes the greatest part of the serum lipids and about 70 per cent of the β-globulin where among others the serum lipids are supposed to occur, without any appreciable change in the other protein components of the serum (2, 7). If the agglutination phenomenon in rheumatoid arthritis

[1] An abbreviated report on the results presented in this paper was given at a meeting of the Finnish Rheumatologists in Helsinki, April 1949.

is due to a non-specific serum factor, it seems likely that this factor would be found in the α-and/or β-globulin fractions of the sera, and accordingly it could probably be removed with bentonite, while the specific agglutinins would not.

MATERIAL AND METHODS

Sera. — The sera used were sera of patients with rheumatoid arthritis tested for streptococcal agglutination, Widal positive sera tested for agglutination with *S. paratyphi B*, some antibacterial immune sera tested with the homologous antigens (*S. paratyphi B*, *S. typhi*, *B. abortus Bang* and *B. proteus* X19), and some blood donors sera tested for isoagglutinins. All the sera except the blood donors sera were inactivated for half an hour at 56° C.

Titration Methods. — To test for streptococcal agglutination the sera were diluted 1: 10 and serial twofold dilutions were made in 1.0 ml of saline. The titrations were performed with living streptococci according to a previous method (3).

The Widal sera and the antibacterial immune sera were titrated using the same dilutions in 0.5 ml of saline and using 0.5 ml of antigen. For the Widal sera H-antigen of *S. paratyphi B* was used. For the anti-*S. typhi* and anti-*S. paratyphi B* immune sera also H-antigens of the respective organisms were used, and for the anti-*B. abortus* Bang and anti-*B. proteus* X19 immune sera O-antigens of the respective organisms. When the H-antigens were used, the mixtures of antigen and serum were incubated for two hours at 37° C and read immediately. When the O-antigens were used, the mixtures of antigen and serum were incubated for two hours at 56° C and read after 20 hours at room temperature.

The sera from blood donors were titrated starting with a dilution of 1: 8 and followed by twofold dilutions in 0.2 ml of saline. To each tube 0.2 ml of a one per cent human red cell suspension (heterologous cells) was added. The results were read after one and a half hours at room temperature.

Absorption with Bentonite — The bentonite absorption was performed according to a previous paper (7) but instead of using tenfold dilutions, five fold dilutions were used for all sera except the blood donors sera, where a fourfold dilution was employed. In this manner the final dilution of the absorbed sera was 1: 10 (for

the blood donors sera 1: 8) as was the starting dilution used for untreated sera. Instead of the bentonite dilutions used previously, 0.25, 0.5, 0.75 and 1.0 per cent bentonite suspensions were used. The *p*H of the sera was not adjusted before absorption.

The titration endpoints were in all cases estimated according to the last clear one plus (+) reaction, and the titer was expressed as the reciprocal of the serum dilution before adding antigen.

All titrations of one serum were made at the same time.

RESULTS

In tables 1—3 the results are given of the agglutination titrations with the Widal sera, the antibacterial immune sera and the blood donors sera respectively before bentonite treatment and after treatment with different concentrations of bentonite. (Tables 1 to 3.)

TABLE 1

AGGLUTINATION TITERS OF FOUR DIFFERENT IMMUNE SERA BEFORE AND AFTER TREATMENT WITH DIFFERENT CONCENTRATIONS OF BENTONITE SOL

Serum	Untreated	Treated with Bentonite			
		0.25	0.50	0.75	1.00
S. typhi	1280	640	160	160	40
S. paratyphi B..	5000	5000	1280	640	320
B. abortus Bang	640	640	640	320	160
Proteus X19....	10000	10000	5000	2560	1280

From the tables it can be seen that the bentonite concentrations 0.25 and 0.5 per cent leave the agglutination titers nearly unchanged. The two higher concentrations of bentonite produce a decrease in the titer, which apparently is due to loss in antibodies. This is in accordance with the previous experiments where the same changes in the antistreptolysin titer could be seen (7). From these control experiments it thus appears that the bentonite treatment does no tappreciable affect the agglutinins in the sera if bentonite is used in suitable amounts. Similar experiments, with similar results although not recorded here, were performed also with syphilitic seropositive sera, using the Wassermann and Kahn tests.

TABLE 2

AGGLUTINATION TITERS OF SOME WIDAL POSITIVE SERA BEFORE AND AFTER TREATMENT WITH DIFFERENT CONCENTRATIONS OF BENTONITE SOL

Serum	Untreated	Treated with Bentonite			
		0.25	0.50	0.75	1.00
4/49	160	160	80	40	20
5/49	1280	640	320	320	160
7/49	640	320	160	160	80
14/49	320	320	80	80	40
3—1/49	320	160	80	40	20
4—1/49	160	40	20	10	10
7—1/49	320	320	160	80	40
10—1/49	320	160	80	40	40
9—2/49	80	80	20	10	10
17—2/49	160	80	80	40	40
20—2/49	40	40	20	10	—
32—2/49	160	80	80	40	40

TABLE 3

HEMAGGLUTINATION TITERS OF SOME GROUP A, B AND O BLOOD DONORS SERA BEFORE AND AFTER TREATMENT WITH BENTONITE

Serum	Untreated	Treated with Bentonite			
		0.25	0.50	0.75	1.00
A— 1	256	256	256	64	64
A— 2	64	64	64	32	32
A— 3	2048	1024	512	256	256
A— 4	128	128	64	64	32
A— 5	256	128	64	64	64
A— 6	32	64	32	16	16
A— 7	128	256	128	64	64
A— 8	256	128	128	64	64
A— 9	256	128	64	32	32
A—10	128	128	64	64	64
A—11	128	64	64	64	64
B— 1	128	128	64	32	16
B— 2	128	128	64	64	32
B— 3	256	128	64	64	64
B— 4	64	32	32	16	16
B— 5	128	64	32	32	32
O— 1	128	256	128	128	128
O— 2	64	32	16	16	16
O— 3	32	16	8	8	8

TABLE 4

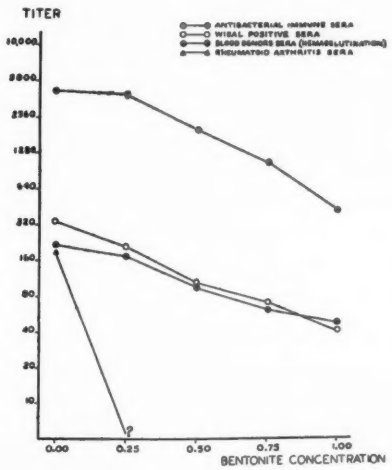
STREPTOCOCCAL AGGLUTINATION TITERS OF SOME RHEUMATOID ARTRITIS SERA BEFORE AND AFTER TREATMENT WITH DIFFERENT CONCENTRATIONS OF BENTONITE

Serum	Untreated	Treated with Bentonite			
		0.25	0.50	0.75	1.00
1	1280	—	—	—	—
2	640	20?	—	—	—
3	320	—	—	—	—
4	320	—	—	—	—
5	160	20?	—	—	—
6	160	—	—	—	—
7	160	—	—	—	—
8	160	—	—	—	—
9	160	—	—	—	—
10	160	—	—	—	—
11	80	—	—	—	—
12	80	—	—	—	—
13	80	—	—	—	—
14	80	—	—	—	—
15	80	—	—	—	—
16	80	—	—	—	—
17	40	—	—	—	—
18	40	—	—	—	—
19	40	—	—	—	—
20	40	—	—	—	—
21	40	—	—	—	—
22	20	—	—	—	—

Table 4 shows the results obtained in the same manner with sera of patients with rheumatoid arthrithis using living streptococci as antigen (Table 4).

In all cases the streptococcal agglutination titer dropped from very high levels, in some instances, to less than ten, even after absorption with the lowest concentration of bentonite. No titrations with less diluted sera were made because these results differed so clearly from those obtained with the other sera that it was assumed that the betonite absorbed the agglutinins from the rheumatoid arthritis sera, but not from the other sera. The difference in behaviour between the »agglutinins» against hemolytic streptococci in these sera, and the specific agglutinins against the other bacteria and the

FIGURE I



blood cells is made clear by Fig. 1., where the curves represent the mean of the results from the tables 1—4. (Fig. 1.)

The data presented in figure 1 show that there is a significant difference in behaviour between the Widal positive sera, the antibacterial immune sera and the blood donors sera on one hand, and the sera of patients with rheumatoid arthritis on the other.

DISCUSSION

The purpose of this and earlier papers has been to find out whether the agglutination of living hemolytic streptococci by sera of patients with rheumatoid arthritis is due to specific streptococcal agglutinins or to some non-specific factor or factors in the sera, and whether this agglutination phenomenon can be regarded as a proof for the streptococcal etiology of rheumatoid arthritis.

In previous papers in has been stated that the specifity of this streptococcal agglutination reaction is doubtful (3, 4, 5, 6). The results presented above show that the factor responsible for the agglutination of living hemolytic streptococci is easily removed by bentonite. This is not the case with other specific agglutinins so far studied with this method. Thus it seems unlikely that the agglutination of living streptococci by sera of patients with rheumatoid arthritis is due to specific antibodies.

Wallis (12) is of the opinion that the agglutination of living hemolytic streptococci by sera of patients with rheumatoid arthritis is due to a non-specific increase in »normal» streptococcal agglutinins, and Wager (11) using autoclaved hemolytic streptococci *ad modum* Thulin (9) states that the agglutination is the result of a factor in the sera activating the agglutinating capacity of minimal amounts of normal streptococcal agglutinins, as in the case of the agglutination of sensitized sheep cells according to Waaler (10) and Rose et al. (8). Although the agglutination of living streptococci and autoclaved streptococci can not without further evidence be regarded as fully comparable, it is possible that an agglutination activating factor might be responsible also for the agglutination of living hemolytic streptococci. If that is the case one has to assume that either the normal streptococcal agglutinins or the activating factor is removed by bentonite. As has been mentioned above the electrophoretic analyses of bentonite treated sera show that the serum component most affected is the β-globulin. If it is assumed that the normal agglutinins occur in the γ-globulin fraction one would perhaps be in favour of the latter assumption, that bentonite removes an agglutination activating factor, or some other non-specific agglutinating factor/factors of the sera. It is likely that if serum fractions which have been electrophoretically-separated are tested for their streptococcal agglutinating capacity, either alone, or as an interaction between different fractions, it would be possible to determine in which component of the serum this factor occurs, thus definitely showing the cause of the agglutination of living hemolytic streptococci by sera of patients with rheumatoid arthritis.

SUMMARY

It has been shown in this and in previous papers that

1. Some strains of *Staphylococcus aureus* are agglutinated by sera of patients with rheumatoid arthritis to nearly the same extent as hemolytic streptococci.

2. The capacity of these sera to agglutinate staphylococci and streptococci dissappears if the sera are absorbed with staphylococci, although experiments with specific immune sera does not prove any definite similarities between streptococcal and staphylo-

coccal antigens. However, absorption with streptococci did not cause disappearance of staphylococcal agglutinins.

3. No clear parallelism could be proved between the occurence of streptococcal agglutinins and increased antistreptolysin titer on one hand, or between occurrence of staphylococcal agglutinins and increased antistaphylolysin titer on the other.

4. The capacity of sera of patients with rheumatoid arthritis to agglutinate living hemolytic streptococci disappears if the sera are treated with bentonite. In all cases of specific agglutination so far tested there was no such total disappearence of the agglutinins after bentonite treatment.

The reason for this serological reaction has been discussed. It has been suggested that the agglutination of living hemolytic streptococci by sera of patients with rheumatoid arthritis is caused by a non-specific factor (or factors) in the sera, probably occuring in the α-and/or β-globulin fraction, and accordingly that this agglutination can not without further evidence be regarded as proof for the streptococcal etiology of rheumatoid arthritis.

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FROM THE DEPARTMENT OF PHARMACOLOGY, UNIVERSITY OF HELSINKI

TOXICITY OF SODIUM SALICYLATE AND AGE

by

M. PAASONEN

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As early as 1909 (10) Moore suggested that the skin might be a more accurate index by which to calculate a dose of medicine than body weight. According to him, younger people are more resistant to doses calculated in relation to their body weight than older people, partly because of proportionately larger area of their skin. This has been proved true of many substances, but the results obtained have varied considerably in different animals and have sometimes been completely contradictory. It seems impossible to generalise about the effects of drugs according to age and size, as each has its own characteristics (3). It would be interesting to know how far younger animals were resistant to a given substance, and whether their resistance could be correlated with the surface area of their skin; whether this were obvious in the case of antipyretics, where the physical pharmacological relations occur mainly on the skin surface. Madisson (9) when examining a small number of rabbits, noticed incidentally that smaller rabbits were obviously more resistant to toxication from sodium salicylate than older and larger ones. Brownlee noticed a similar phenomenon in mice and rats when he examined the toxicity of other antipyretics (2). The fact that the majority of fatal cases of salicylate poisoning were children under five can be explained by inadequate medical care and the proneness of children of that age to eat anything and everything (7). In fact, it is generally held that the toxic dose is larger in proportion for children than for adults (4).

Since salicylic acid, its salts and derivatives are widely used, we thought it advisable to examine the tolerance to sodium salicylate in guinea-pigs, rats, and mice of different ages.

METHODS

The tests were made on male guinea-pigs, male white mice, and non-pregnant female white rats (Vistar strain). The animals in the various age groups had grown up under similar conditions. We do not give the ages of the guinea-pigs and mice as we did not know them exactly, but their body weight was in direct proportion to their age. The heaviest in each group were considered as young adults; of these the guinea-pigs were about 8—10 months old, the mice about 5 months, and the rats exactly 8 months. The younger group of rats were about 6 weeks old. The animals received ordinary food both before and during the tests. They were kept at room temperature (16—20 degrees C.). Sodium salicylate was administered subcutaneously and the injections were given in the afternoon. The last observation of the animals was after 48 hours. The tests on the guinea-pigs and mice were made between February and May, those on rats in October. The concentrations used, made in distilled water, were 25 per cent for the guinea-pigs, for rats 10 per cent and for mice 5 per cent. The areas were calculated using Meeh's formula, according to the average weight of each animal, i.e. area $8.5 \sqrt[3]{(\text{weight})^2}$

RESULTS

The lethal dose for guinea-pigs reaches its maximum during the period of quickest growth (Table 1). For adults it was lower and lowest for those weighing under 150 g. In the youngest group the

TABLE 1

Body Weight g	Sodium Salicylate mg/kg							LD_{50}	
	400	600	800	900	1000	1100	1200	mg/kg	mg/cm²
60—149.....	1/6*	6/9	3/3					520	0.282
150—400.....		0/1	0/2	0/3	1/9	4/8	4/6	1100	0.860
550—850.....		1/5	2/8	5/8	4/4			800	0.855

* Mortality ratio: No. killed/No. in groups.

heaviest animals seemed to have more than average resistance, but owing to the limited number of animals used it was not thought necessary to subdivide the group. In the other two groups the distribution was pretty even. Where the area was calculated from average weight there was no difference in the fatal dose between the two heavier groups, when the dose was estimated to a square unit of the animal. On the other hand, in the youngest group the dose varied considerably.

TABLE 2

Body Weight g	Age	Sodium Salicylate mg/kg						LD 50	
		500	600	700	800	900	1000	mg/kg	mg/cm²
80—110	6 weeks			0/6	4/12	5/6	7/7	820	0.450
180—220	8 months	0/6	2/6	6/10	7/7			650	0.480

The results from rats (Table 2) were similar to those for guinea-pigs in the two older age groups. In the experiments the tendencies were similar. However, the fatal dose per area unit was even larger for older than for younger rats.

TABLE 3

Body Weight g	Sodium Salicylate mg/kg							LD_{50}
	300	400	500	600	700	800	900	mg/kg
6—12	1/8	6/12	8/10	6/6				400
13—18		0/5	3/9	12/14	6/6			530
19—25		0/3	0/8	6/12	7/10	7/7		600
>25			0/6	1/9	6/14	10/12	10/10	700

The results obtained for mice (Table 3) differed from those for the other groups in so far as toxicity was not greater in the oldest group compared with the next but oldest, but the lethal dose increased in all groups with age. Therefore, the dose was largest for the oldest group, in each case.

The toxication affected the older and younger groups of guinea-pigs differently, the younger ones obviously became weak and limp, the older ones often had tonic or clonic cramps. This was not noticeable in the other animals, at least not in the same degree.

It is difficult to compare those pharmacological effects of the

salicylates which may be considered therapeutic. The hyperthermia required for the study of antipyretic characteristics cannot be induced with equal success in animals of different ages. Analgesic properties, on the other hand, are measured by skin sensitivity, but great changes take place in the skin with age. Any comparison of the reactions by these criteria was therefore abandoned.

ESTIMATION OF THE FATAL DOSE

The method of calculating the fatal dose is illustrated by a simple graph (Figs. 1, 2, and 3).

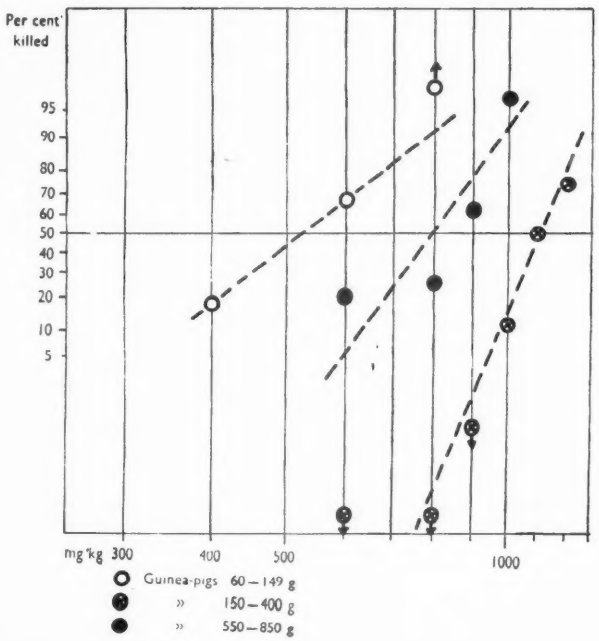

Fig. 1.

The graphic method presupposes that the mortality ratio curve changes, as the dose is increased, in such a way that the probit of the mortality ratio is proportional to the logarithm of the dose. The usual probit analysis is made on the same assumption. If this assumption is correct, the mortality curve can be represented by an approximately straight line in a co-ordinate where the dose scale

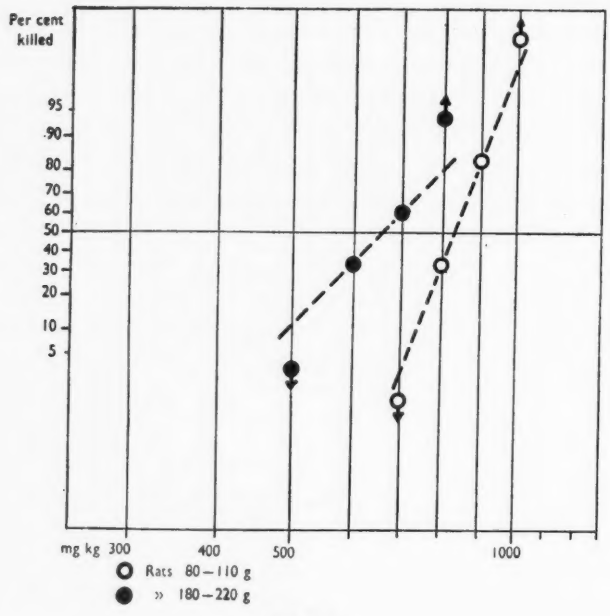
Per cent killed
95
90
80
70
60
50
40
30
20
10
5
mg kg 300
400
500
1000
Rats 80–110 g
» 180–220 g

Fig. 2.

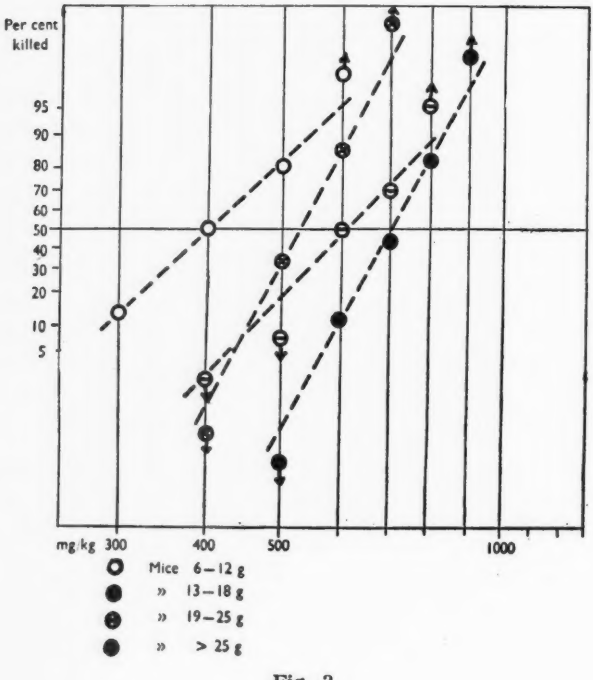
Per cent killed
95
90
80
70
60
50
40
30
20
10
5
mg/kg 300
400
500
1000
Mice 6–12 g
» 13–18 g
» 19–25 g
» > 25 g

Fig. 3.

is a logarithm and the mortality ratio scale a probity one. In the diagrams the 0 per cent and 100 per cent cases are indicated by arrows. The observation points were marked on the minimum or maximum working probity value, as it is called, obtained from the appropriate tables. The fatal doses were read off from the graph straight away. It is clear that these estimations differ very little from estimations obtained by artithmetical calculation, and such slight differences are here of no essential importance. Calculation of errors was also considered not necessary.

DISCUSSION

No satisfactory explanation exists of the resistance or sensibility of growing creatures to certain drugs. Various substances which have a narcotic effect on the central nervous system, morphine being the most typical, form a special group by themselves. As we know this is most toxic in very young children. According to Gibbs (6) myelinisation of the central nervous system and increasing resistance to morphine take place simultaneously in the rat. Although the irritation of the central nervous system, especially the disturbance of the respiratory centre has been considered the most important initial cause of toxication symptoms caused by salicylic acid (8), it is unlikely, however, that the variations of the fatal dose can be attributed entirely to the variations in the reaction of the nervous system. That young animals are more resistant to certain substances may be explained by their more rapid metabolism which enables them to destroy or eliminate the poison more quickly. Again the area of the skin is considered a better criterion of metabolism than body weight. But the elimination of salicylates through the skin is insignificantly small. For older growing guinea-pigs and rats, and young adult ones, the area of the skin seems a better criterion on which to base the fatal dose than the body weight of the animal. It has been claimed (1) that the secretion of salicylates in the urine decreases with age. In mice the adult individuals are even more resistant than growing ones. Sodium salicylate is a pretty irritating substance, and one would suppose that its chances of destroying the tissues in a smaller animal were proportionately greater. In that case the absolute size and the fatal dose would be directly proportionate.

If we can apply to human beings conclusions drawn from results obtained with guinea-pigs and rats, we should expect that the resistance of more developed children would be greater while that of the youngest children would be possibly smaller than what has been calculated. From the point of view of salicylate therapy a successful comparison of the doses for antipyretic and other treatment of test-animals of different ages would be of value in itself. Of late the doses recommended for children have been calculated on the same basis as for adults, namely, according to weight (5).

SUMMARY

Sodium salicylate was injected subcutaneously into growing and young adult guinea-pigs, rats and white mice. The LD50 for older growing guinea-pigs and rats was obviously greater in proportion to their weight than for adults. When the dose was calculated on the basis of skin area it was just as large for guinea-pigs, and showed less variation for rats. The LD for the smallest guinea-pigs was lowest of all. For mice no similar increase in resistance apparent during the period of quickest growth could be noted.

I wish to thank Professor A. Vartiainen for suggesting the subject of this study and for his helpful criticism.

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FROM THE DEPARTMENT OF SEROLOGY AND BACTERIOLOGY, UNIVERSITY OF HELSINKI

PHYTAGGLUTININS PRESENT IN MARASMIUS OREADES

by

JAAKKO ELO and EERO ESTOLA

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In our investigation into the phytagglutinins present in fungi (1), one of the extracts — from the fungus *Marasmius oreades* — proved to agglutinate B cells considerably more strongly than any other cells. As we then had only one fungus individual at our disposal we were unable to check the result or to carry out further experiments on the nature of these agglutinins.

In continuation studies we were able to dispose of 4 fungal samples grown at different sites. One of them, which had been stored for 2 years dried, did not agglutinate at all. The remaining 3 fresh strains gave mutually identical results.

A 10 per cent saline solution was prepared as before by pounding 1 part of fungus and 9 parts of saline solution in a mortar into a homogeneous suspension. This was allowed to stand for an hour in the incubator (37°C). The suspension was centrifuged and the supernatant used for the investigations.

Agglutination and absorption experiments were carried out at room temperature in the normal way. As can be seen from Table 1, the agglutinin present in the extract is of the panagglutinin type, as were the other phyt agglutinins. The red cells possessing the B property agglutinate in the extract and absorb it most strongly, the A_1 cells most weakly. A_2 and O cells agglutinate and absorb equally strongly. A blood sample known to be of genotype BB prove no different from the other B cells in its reactions.

TABLE

TITRE OF 10 PER CENT SOLUTION

	Unabsorbed						After A_1					
	A_1	A_2	A_1B	A_2B	B	O	A_1	A_2	A_1B	A_2B	B	O
1/1	—?	+++	+++	+++	+++	+++	—	—?	+	+++	+++	—
1/2	—	++	++	+++	+++	++	—	—	(+)	++	+++	—
1/4	—	(+)	+	+++	+++	—	—	—	—?	++	++	—
1/8	—	—	+	+++	+++	—	—	—	—	+	+	—
1/16	—	—	(+)	++	++	—	—	—	—	(+)	+	—
1/32	—	—	(+)	+	+	—	—	—	—	—?	(+)	—
1/64	—	—	—?	+	+	—	—	—	—	—	—?	—
1/128	—	—	—	(+)	+	—	—	—	—	—	—	—
1/256	—	—	—	—	(+)	—	—	—	—	—	—	—
1/512	—	—	—	—	—?	—	—	—	—	—	—	—

TABLE 2

AGGLUTINATION TITRE OF M. OREADES AT DIFFERENT TEMPERATURES

T°C	Cells	Dilution of Fungal Extract								
		1/1	1/2	1/4	1/8	1/16	1/32	1/64	1/128	1/256
2°	A_1	++	+	(+)	—?	—	—	—	—	—
	A_2	+++	+++	++	+	—?	—	—	—	—
	B	+++	+++	+++	+	+	+	(+)	—?	—
	O	+++	+++	+++	(+)	—?	—	—	—	—
20°	A_1	+	(+)	—	—	—	—	—	—	—
	A_2	+++	+++	(+)	—	—	—	—	—	—
	B	+++	+++	+++	++	+	(+)	(+)	—?	—
	O	+++	+++	—?	—	—	—	—	—	—
37°	A_1	—?	—	—	—	—	—	—	—	—
	A_2	+	(+)	—	—	—	—	—	—	—
	B	+++	+++	+++	+++	+++	+++	++	+	—
	O	+	—?	—	—	—	—	—	—	—

The influence of temperature on the agglutination ability of the extract can be seen from Table 2.

The agglutination capacity of B cells is found to remain constant with rising temperature while the titre of the other cells declines, the extract thus becoming more B-specific.

The fungal extract was diluted until the A and O cells employed

1

OF MARASMIUS OREADES

Absorption with Half Volume of Cells

A_2						O						A_1B, A_2B or B					
A_1	A_2	A_1B	A_2B	B	O	A_1	A_2	A_1B	A_2B	B	O	A_1	A_2	A_1B	A_2B	B	O
—	—	(+)	++	++	—	—	—?	—?	+	+	—	—	—	—	—	—	—
—	—	—?	+	+	—	—	—	—	—?	(+)	—	—	—	—	—	—	—
—	—	—	—?	(+)	—	—	—	—	—	—?	—	—	—	—	—	—	—
—	—	—	—	—?	—	—	—	—	—	—	—	—	—	—	—	—	—
—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—

as controls, no longer agglutinated. 340 random samples were examined with the extract thus obtained; 134 of them belonged to blood group A, 130 to O, 46 to B and 30 to AB. All 76 samples of groups B and AB gave strong reactions; those of groups A and O failed to agglutinate.

SUMMARY

The results of continuation investigations into the phytagglutinins present in the fungus Marasmius oreades are reported. The extract agglutinates cells containing the B-property considerably more strongly than other cells, and the absorption capacity of these cells, accordingly, is also greater than that of A and O cells.

A blood sample known to be of genotype BB prove no different from the other B cells in its reactions.

The agglutinin contained in the extract is of the panagglutinin type, and it becomes increasingly B-specific with rising temperature. From among 340 random samples, all 76 in groups B and AB could be distinguished by means of the extract.

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FROM THE DEPARTMENT OF PHARMACOLOGY, UNIVERSITY OF HELSINKI

DIHYDROERGOTAMINE AND HISTAMINE TOXICITY

by

DANIEL ECKERT and M. PAASONEN

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In order to eliminate toxic effects of histamine the organism has at its disposal histaminolytic ability, one component of which, histaminase, histamine decomposing enzyme, has been known for a long time [Best and McHenry (1)]. The abatement of the biological effects due to histaminase is, however, so slow that the elimination of the effects of histamine cannot be fully explained by referring to it. Thus, Dale and Richard (4) a long time ago paid attention to the antagonism between histamine action and the adrenal gland, and then nearest to the hormone of the medulla adrenaline. It has been stated that after injection of histamine the adrenal gland increases the adrenaline secretion [Dale (3), Burn and Dale (2), Mackay (9)]. Histaminaemia caused by adrenaline was first stated by Eichler and Barfuss (6) and later by Staub and Baur (14), who showed that adrenaline liberated histamine in physiological circumstances in man and rabbit.

Studies on the effects of sympathicolytics on the reactions caused by histamine are interesting from the point of view of the adrenaline-histamine antagonism. According to Ganter and Schretzenmeyer (7) ergotoxine causes no alterations in the histamine-produced blood response while ergotamine has a slightly sensitising effect on it. Smith (13), on the other hand, noted that in the dogs ergotamine causes sensitisation to histamine to such an extent that 1—2 mg per kg produced a fatal shock, while in normal dogs the lethal dose is ca. 10 mg per kg of the body weight. When studying

Our thanks are due to Messrs. Sandoz A. G. for kindly supplying us with dihydroergotamine.

the effect of dihydraergotamine (DHE 45, Sandoz) on the histamine-produced blood pressure reaction in anesthetised rabbit Eckert and Vartiainen (5) showed that both small (0.02—0.04 mg/kg) and large (0.4 mg/kg) doses of dihydroergotamine as a rule sensitivised the blood pressure effects of histamine, although the sensitising characters of small and large doses were different. In supplementary sense the question is of interest, whether dihydroergotamine has any effect on the lethal dose of histamine on guinea-pigs and whether there exists any relation between the dihydroergotamine dose and the lethal dose of histamine.[1]

Methods. — 48 guinea-pigs, male and non-gravid female, the weight of which varied from 350 to 450 g, were anesthetised with 25 per cent urethane solution (2 g of urethane per kg). One hour or one hour and a half after the administration of urethane the jugular vein was prepared forth and furnished with canule for the infusion of the histamine solution. The preparation took ca. 5 minutes. To the animals to which dihydroergotamine was administred, it was given as heart punctions properly diluted immediately before the preparation of the vein. The histamine solution 1: 40,000 was prepared by dissolving acid histamine phosphate in physiologic NaCl solution, in which a drop of 5 per cent heparine had been added. 0.5 ml of the solution 1: 40,000 (0.0125 mg of histamine base) was infused each minute until the breathing ceased.

TABLE 1

Number of Animals n	Dihydro-ergotamine mg/kg	Histamine LD Mean		Body Weight Mean g	$\frac{\Sigma d^*}{n}$	
		mg/Animal	ml/Animal		Histamine LD ml/Animal	Body Weight g
12	—	0.12	4.8	386	1.64	23
12	1.0	0.04	1.6	370	0.35	21
9	0.1	0.07	2.7	396	0.68	18
7	0.02	0.08	3.9	371	1.27	15
8	0.01	0.12	4.9	361	1.05	27

* Σd = The sum of the deviations from the mean value of various lethal doses and body weights.

[1] While the paper was in the press, we learned that Corelli was able to prevent fatal histamine shock in most his rabbits by pretreatment with 2mg dihydroergotamin intravenously (Rass. Neurol. Veg. 1950: *8*:101; cit. Rothlin, E., and Bircher, R. in Progress of Allergy III. S. Karger, Basel, New York 1952).

Results. — When examining the effect of histamine injected in the manner described above on normal guinea-pig it is noted (Table 1) that the lethal dose is of the same quantity category as the LD mentioned in literature, when histamine was administred i.v., or ca. 0.3 mg/kg (8, 12). The volume injected in normal animals was 4.8 ml in average, which means that death followed after ca. 9.5 minutes. When about one half of the lethal dose was infused, with some animals a condition was reached where the breathing did not seem to become essentially more difficult during the following minutes, but death followed only later when the breathing suddenly ceased. Another kind of attitude was represented by the animals, around 2/3, whose breathing became more difficult evenly until the end. The animals that had received 0.1 and 1.0 mg/kg of dihydroergotamine reacted strongly to histamine, while death followed after 3 to 3.5 minutes. Strong bronchospasm was caused generally immediately from the first dose of histamine, while the condition grew worse evenly. On animals that had received 0.02 mg/kg of dihydroergotamine, histamine had the same effect, although not as clearly. Doses of 0.01 mg/kg could not be proved to have any effect. The variations of the LD of histamine and of the body weight in the different groups are demonstrated by $\frac{\Sigma d}{n}$ which is the mean value of the deviations of the different animals in each group. Guinea-pig is very resistent to dihydroergotamine (10) and doses concerned cannot be considered to have as such any toxic effects (11). No differences could be found to exist between the different sexes.

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FROM THE DEPARTMENT OF PHARMACOLOGY, UNIVERSITY OF HELSINKI

TOXICITY OF SALICYLAMIDE

EFFECT OF COMBINING SALICYLAMIDE WITH SODIUM SALICYLATE AND ACETYL-SALICYLIC ACID ON THE LETHALITY OF THESE SUBSTANCES FOR WHITE MOUSE

by

M. PAASONEN and T. PELTONEN

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Although salicylates have been widely used as drugs for a long time, they are known to have side-effects which often greatly limit the possibility of using them. Because of this, several combinations of them have appeared, expected to be less toxic. Salicylamide, a drug discovered no less than a hundred years ago, is a derivative of salicylic acid, which has been practically forgotten until quite recently. It differs in its pharmacological effects from other salicylic derivatives in many respects. Its narcotic properties were its most important ones which H. H. Myer (9) discovered in frogs as early as 1901. Ichniowski and Heuper (7) studied the toxicity of salicylamide in both acute and chronic tests on rats and found that its effects *in vivo* differed essentially from those of other salicylic combinations. It had obviously a depressing effect on the central nervous system, but did not cause any morphological changes in the tissues or blood, nor did it alter the coagulation of the blood. Hart (6) and Litter, Moreno and Donin (8) noted a similar depressive and paralysing effect. According to the latter, this is a phenomenon common to guinea-pig, rabbit, dog, cat and mouse. Doses of 0.2 to 0.5 g/kg i.p.produced narcosis for 1 to 2 hours, and doses of 1 to 2 g/kg were lethal after between half and one hour because of respiratory arrest. Even small doses noticeably hindered respiration. The action of the heart was weakened only after very

large doses. Again the smooth muscles of the intestine were paralysed and uterine contraction caused by adrenaline prevented in rabbits. From this it was recognised that salicylamide obviously differed from the salicylates, which are essentially irritants and cause death by central irritation and convulsions. The authorities just quoted showed that salicylamide is indicated »in rheumatoid conditions and is better tolerated than salicylates». It further differed from salicylates in shortening the prothrombin period. Seeberg, Hansen and Whitney (11) found only 1/4 to 1/8 of the amount in the serum after the administration of the salicylamide as compared to the quantity produced by an equal dose of salicylate. The low serum concentration of the former, however, was due to its rapid diffusion in the tissues. Spühler and Marti (12) also found that the smallness of the amount of salicylamide discovered in the blood was due to its great capacity for combining with the proteins in the blood.

Taking into account these characteristics of salicylamide and the fact that, according to information available, it must be considered a clinically valuable drug in the diseases where salicylates are used, it was thought to be worth while to find out whether, in practise, it would be possible to reduce the symptoms of salicylic intoxication by giving salicylamide and common salicylates at the same time. Sedatives, such as barbiturates, morphine and bromides (3.4), have been recommended for the irritation and convulsions caused by the salicylates. On the other hand, sedatives have been said to be dangerous in case of salicylate intoxication (5). In the investigation reported in this paper we mainly tried to find out, both by acute and chronic tests, whether the lethal dose for a white mouse was larger if salicylamide and sodium salicylate or acetyl-salicylic acid were given simultaneously.

METHODS

White mice weighing 20 to 30 g were used, and the tests made at room temperature (16 to 20°C). The injections were given subcutaneously. The salicylamide was prepared by shaking methyl salicylate and ammonia together (1). By determining the melting point we proved that the substance obtained was quite pure. The sodium salicylate was a 5 per cent solution in water; the acetyl-

salicylic acid and salicylamide were 5 per cent water suspensions, 2 per cent gum arabic being the suspending material. In the acute tests the animals were observed for two days. The mice were fed freely on rolled oats, white bread, milk and water before and during the test. In the test where salicylamide and sodium salicylate or acetyl-salicylic acid were administered, the salicylamide was given first and, when its sedative effect was clearly obvious, in about half an hour, one of the other drugs. They were injected into the skin of the back, as far from each other as possible.

The lethal dose was calculated by the simple graphic method (Fig. 1) explained in a previous paper (10). It is clear the results differ only very slightly from those obtained by calculation. It was not considered necessary to make any calculation of errors.

RESULTS

TABLE 1

mg/kg	Sodium Salicylate	Acetyl-Salicylic Acid	Salicylamide
400	0/5[1]		
500	4/24		
600	9/20	0/3	
700	16/20	2/8	
800	10/10	2/10	0/16
900		8/18	1/ 7
1000		12/15	2/13
1100		10/10	4/10
1200			11/20
1300			7/10
1400			15/16
LD50	610	880	1150

[1] Mortality ratio: No. killed/No. in groups.

Table 1 shows the average lethal doses of the substances tested when given separately. The average lethal dose for sodium salicylate is 610 mg/kg, while that for salicylamide is almost twice as much. Acetyl-salicylic acid comes in between the two.

There are two main groups in table 2 where the mice were given 600 mg/kg of salicylamide in the first and 1000 mg/kg. of

TABLE 2

mg/kg	Salicylamide 600 mg/kg (52 per cent of LD50)		Salicylamide 1000 mg/kg (87 per cent of LD50)
	Sodium Salicylate	Acetyl-Salicylic Acid	Sodium Salicylate
200			4/14
300	2/27		12/20
400	6/15	3/10	8/8
500	11/15	11/19	6/6
600		10/10	
LD50	440	470	270
% of LD50 without salicylamide (see text)	72 52+72=124	53 52+53=105	44 87+44=131

it in the second. The LD50 of sodium salicylate injected half an hour later is 440 mg/kg in the first group and 270 mg/kg in the second. The corresponding figure for acetyl-salicylic acid is 470 mg/kg in the first group. These LD50 figures are 72 per cent, 44 per cent and 53 per cent of the corresponding figures for the same injections without salicylamide. A salicylamide dose of 600 mg/kg is 52 per cent of the fatal dose, while one of 1000 mg/kg is 87 per cent of it. From these figures it appears that the doses the animals tolerated were slightly larger in the case of sodium salicylate than one would expect from arithmetical calculation alone. In other words, the salicylamide dose in percentages of its LD50 plus the DL50 of the sodium salicylate after the administration of calicylamide in percentages of its LD50 without any salicylamide, should be 100. In the first group this figure is 105 for acetyl-salicylic acid and 124 for sodium salicylate. The relatively largest dose tolerated was 131 for sodium salicylate and salicylamide, in the second group when the salicylamide dose was very near the fatal level.

In the chronic test (Table 3), over a period of 16 days gradually increased doses of sodium salicylate in the first group, salicylamide in the second group and both in the third group were injected into mice. Each dose was injected on four days. The first dose was about

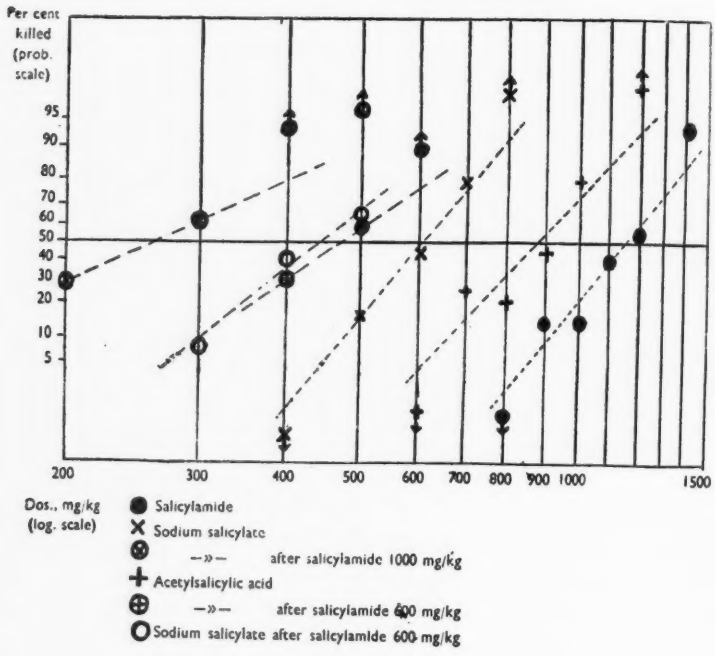

Fig. 1.

TABLE 3

					No. of Deaths in Each Period				Mortality Ratio
Periods of Four Days each	1	2	3	4	1	2	3	4	
Injections per Day	1	1	2	2					
Sodium salicylate ..	300[1]	400	300	400	—	—	—	1	1/30
Salicylamide	560	760	560	760	—	2	7	13	22/30
Sodium salicylate +	150+	200+	150+	200+					
Salicylamide	280	380	280	380	—	1	2	12	15/30

[1] Dose mg/kg.

half the LD50 for the first two groups and for the third group it was one fourth of the corresponding LD50 of each substance. The doses were increased in equal proportions, given in the table. For the first eight days the injections were given once a day and after that twice a day. Mortality was highest among those which got

salicylamide (22/30), obviously smaller among those which got both substances, and almost nil among those which got sodium salicylate (1/30). It was discovered, pathologically, that the mice which died from the salicylamide all had enlarged, yellowish livers.

In the chronic test we can hardly consider the salicylamide doses used as tolerable as the sodium salicylate one because, for one thing, even after only one salicylamide injection a test animal often died on the third or fourth day, and this was not taken into account in calculating the LD50. In many of the mice, particularly those that died later on, an internal ear defect developed after the salicylamide was injected manifesting itself very noticeably by the mouse rolling in one direction. The dose in these cases had always been near the fatal amount. With sodium salicylate similar symptoms appeared in two mice only, and even late mortality was rare. This may be explained by the fact that salicylamide resorbs readily in brain matter (11). Although the same salicylamide was used in the last tests we were surprised that this phenomenon did not appear. In truth, we were not certain whether the test animals were of the same stock, and the earlier tests had been made in summer while the last were in autumn. Nor did we get rolling mice in the chronic test.

DISCUSSION

The largeness of the lethal dose of salicylamide cannot be explained with certainty. As their solubilities are similar, one would expect the resorption properties of acetyl-salicylic acid and salicylamide to be similar. Perhaps the facility with which salicylamide combines with the proteins of the plasma (12) decreases its »effective» concentration. The fact that the animals which were given salicylamide died after a longer time than those that were given sodium salicylate supports the belief that the effects of the first last longer. Chabamier and others (2) state that »only the unbound salicylate passes the kidney with the clearance of approximately the same as that of urea», and in accordance with this one would expect salicylamide to be eliminated more slowly than the other salicyl combinations. In the chronic test, in particular, one would think this ought to be one factor that would increase the toxicity of salicylamide. In human beings, on the

other hand, secretion of sodium salicylate and salicylamide into urine are equally rapid (12). The middle ear defects also speak for there being an organic brain injury. Of what importance this may possibly be clinically we cannot know for certain because experience of it is slight. It is commonly held, in fact, that ringing in the ears and other side-effects are relatively rare after the administration of salicylamide (13).

It appears from the combined test that salicylamide on the one hand and sodium salicylate or acetyl-salicylic acid on the other, given simultaneously, do not, to any great extent at least, modify the toxic effects of each other in the dosages mentioned. It looks as if the fatal doses were larger than had been calculated when sodium salicylate was administered after salicylamide, but this increase of 20 to 30 per cent is not yet final proof. Even the resorption apparently differs when water-soluble and insoluble substances are in question.[1] This fact is borne out when we can see no increase in the calculated dose of acetylsalicylic acid after doses of salicylamide. The curves are based on a rather restricted animal material, but it seems that salicylamide and the salicylates mentioned do not increase each other's toxicity in fatal doses, but that the reverse may be true especially for sodium salicylate.

We can point to nothing similar between salicylate and salicylamide in the chronic tests. Salicylamide is noticeably more toxic when administered in doses that are relatively equally large. The combined dose remains midway between the other two. Longer chronic tests cannot be made, not if the injections are given subcutaneously, at least. Actually severe necrosis results and this may be partly the reason why the salicylamide animals died.

SUMMARY

The LD50s (average lethal doses) for white mice of sodium salicylate, acetyl-salicylic acid and salicylamide are 610, 880, and 1150 mg/kg respectively when injected subcutaneously.

About half an hour after a salicylamide dose of 600 or 1000 mg/kg the animals tolerated 24 and 31 per cent more sodium

[1] Gum arabic, on the other hand, may also retard the resorption of salicylamide.

salicylate than was calculated. There was no such increase when acetyl-salicylic acid was given after salicylamide in the same way.

When salicylamide was given in relatively the same doses in the chronic test it was clearly more toxic than the sodium salicylate. Given together, sodium salicylate and salicylamide have no effect on each other's toxicity when injected repeatedly.

Salicylamide causes complications more often than sodium salicylate does; these result in the test animal rolling in a specific direction.

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FROM THE INSTITUTE OF OCCUPATIONAL HEALTH, HELSINKI

ERYTHROCYTE AND HAEMOGLOBIN CHANGES DURING PROTRACTED HEAVY MUSCULAR WORK

by

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In the course of years, a considerable number of investigations have been made on changes of the erythrocyte and haemoglobin (Hb) levels in connection with muscular work. Both rises (1, 2) and falls (3, 4) in these values have been reported, whereas in some investigations, no consistent tendency was observed (5, 6, 7). Our attention to these phenomena was stimulated by the »clinical» observation made by one of us (M.K.) that in athletes' training camps, the appearance of symptoms of »staleness», of »overtraining», was regularly associated with a marked fall in the erythrocyte and haemoglobin levels. It seemed that repeated Hb determinations and/or erythrocyte counts might be able to give valuable information of the balance between the fitness of the organism and the stress laid upon it. An opportunity to get further experience of the value of this method was obtained in the National Finnish Lumber Competition, 27—30 November 1951, when a group of selected top class workers took part in a form of »athletics» which undoubtedly is one of the most severe tests of endurance. The present paper is a report of those studies.

MATERIAL AND METHODS

Blood samples were obtained from 7 volunteers, who took part in the National Lumber Competition. The forty participants of the competition were selected with the aid of double trials from a total

of 995 men taking part in the district competitions, and thus they represented a group of well trained athletes. The competition lasted four days: during the first and the last day then men worked for 6½ hrs, whereas the 2nd and 3rd day were half-days, 3¼ hrs each. Blood samples were taken on the day before the competition and during every morning at 6 a.m. before the morning meal and in the evening at 4 p.m., approximately half an hour after the end of the work, before meal. Blood was taken from the tip of a finger. The Hb was determined with a Hellige haemometer, as acid haematin, in controlled illumination and by one analyst. The erythrocytes were counted in a Bürker counting chamber, from 0.0001 cu mm.

The counts were started on six subjects, but as three of these dropped out of the competition at various stages because of exhaustion or accidents, a seventh subject was enlisted on the 2nd day of the competition.

RESULTS

The Hb values of the subjects varied in the first samples between

TABLE

THE AVERAGE CHANGES (± THEIR STANDARD ERRORS) OF THE ERYTHROCYTE AND HAEMOGLOBIN LEVELS DURING WHOLE DAY WORK, HALF DAY WORK, AND NO WORK, AND DURING THE SUBSEQUENT NIGHTS

	Number of Observations	Erythrocytes			Haemoglobin		
		Change Thousands of Cells per cu mm	Falls	Rises	Change g/100 ml	Falls	Rises
(A) Whole day work	10	—479 ± 61	10	0	—0.81 ± 0.27	9	1
(B) Half day work	10	—153 ± 127	6	4	—0.58 ± 0.19	7	0
(C) Whole day rest	9	— 13 ± 70	6	3	±0.00 ± 0.09	3	4
(D) Night after (A)	10	+162 ± 114	3	7	+0.62 ± 0.24	1	7
(E) Night after (B)	11	+321 ± 72	0	10	+0.67 ± 0.25	1	8
(F) Night after (C)	10	+156 ± 78	4	6	+0.42 ± 0.26	1	7

Single underlining means significance at the 5 % probability level
Double » » » » » 1 % » »
Triple » » » » » 0.1 % » »

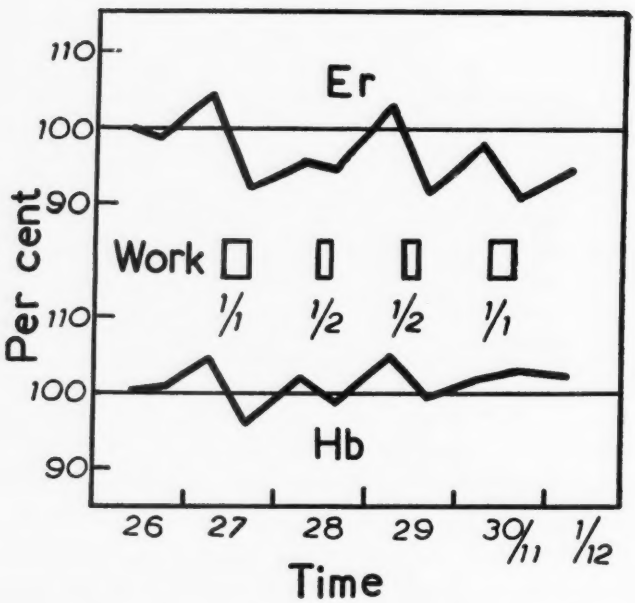

Fig. — The average erythrocyte and Hb value of the competitors throughout the course of the competition. The figures are calculated as percentages of the first morning value of each subject. The period of work is marked with the 1/1 day and 1/2 day columns.

18.4 and 11.8 g per 100 ml, and the range of the erythrocyte counts was correspondingly from 5,490,000 to 3,440,000 cells per cu mm. The subject with the lowest starting Hb and erythrocyte values dropped out of the competition after the first day, because of exhaustion, but otherwise there was no obvious relation between the result in the competition and the level of the blood values.

During the course of the competition, the blood values changed in a more or less regular fashion. In the Table, the results are expressed as the change between two successive observations. The changes are grouped, according to the use of the period over which they took place, in change during a whole day of work (A), during a half day of work (B), during a day without work (C), during a night after A (D), a night after B (E), and a night after C (F). It is observed that a significant *decrease* in both the *Hb and erythrocyte* values occurred *during a whole day of work*, and a significant *rise* of both values took place *during a night following a half day*

of work. In the *Hb* values, a statistically significant *drop* occurred also *during a half day of work, and a rise during a night after a whole day of work,* but in the erythrocyte counts, the scatter was greater and the corresponding tendencies could not be statistically proved.

The averages throughout the course of the competition appear from the Fig. The figures are calculated as percentages of the first morning value of each subject. After dropping off, the values of a competitor are no more included in the average. In Hb values, there was no tendency to a rise or a fall during the competition, and the slight drop in the erythrocyte values is statistically insignificant.

The range of the index was from 0.90 to 1.32. The changes in the index were variable, without any statistically significant tendencies.

No correlation could be observed within this group of competitors *between the performance* in the competition *and the observed changes in the blood values.*

DISCUSSION

The present observations show that in this type of exercise, which probably is one of the most severe forms of protracted muscular work, the Hb and erythrocyte levels of blood fall during a whole day of work, and have a tendency to fall also during a half day of work. During a night after a whole working day, there may occur a further fall or a recovery, whereas after a half working day, the nightly change regularly occurs in the sense of recovery. The present results fall in line with those of Egoroff (5) obtained in marathon race and with those obtained on dogs by Broun (8, 9, 10, 11) and Gurejew (3) or on rats by Dalton and Selye (4). According to Broun, heavy exercise in dogs not used to it causes a destruction of erythrocytes, which again acts as a stimulus for increased erythropoiesis, evidenced by reticulocytosis. An increase in the number of reticulocytes has been observed in connection with heavy exercise also in human subjects (6, 12).

The present results do not incicate, whether the observed changes have been only relative, due to variations in the plasma volume, or whether they indicate a destruction of erythrocytes. Further studies by determining the changes in the total Hb and the plasma volume separately in connection with heavy exercise are desirable.

The determination of the total Hb would be particularly interesting, because a very high correlation has been demonstrated between the amount of total Hb and the physical fitness as tested with the aid of cardiovascular function tests (13, 14). However, a determination of the Hb and erythrocyte values evidently also gives some kind of measure of the ability of the organism to stand exercise. A marked fall of the erythrocyte and Hb values in the work, followed by a further fall during the subsequent night, is not compatible with continuous work at the same rate. In the present work, a progressive change was not observed, perhaps because the 2nd and 3rd day of the competition were only half-days and offered an opportunity to recovery. Additional information on the value of the Hb and erythrocyte changes as an indicator of physical fitness are likely to be obtained either on less well trained subjects or — in trained subjects — by making the men work intensely for successive whole working days.

SUMMARY

1. The daily changes in the erythrocyte and haemoglobin (Hb) levels of seven participants of the National Finnish Lumber Competition were observed throughout the 4 days' course of the competition.

2. In the erythrocyte levels a significant drop of an average of 0.5 million erythrocytes per cu mm occurred during a whole day of competitive work, and a rise of 0.3 million cells was observed during a night following a half-day of work.

3. The Hb concentration showed a significant drop both after a full and also after a half-day. A rise was observed during the subsequent night in both cases.

4. The applicability of Hb determinations and erythrocyte counts to the control of the physiological balance between physical fitness and the exercise required is discussed.

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FROM THE INSTITUTE OF OCCUPATIONAL HEALTH, HELSINKI

EFFECT OF SHORT EXPOSURE TO HIGH ENVIRONMENTAL TEMPERATURE ON WATER DIURESIS IN RAT

by

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It has been observed that the urine flow in hydrated dogs diminishes, if the animals are kept at an elevated ambient temperature (1). This reduction of the urine flow does not occur in animals from which the neurohypophysis has been operatively removed (2). The reduction of the urine flow has been ascribed to a direct influence of the raised temperature on the anterior hypothalamus, which stimulates a release of the antidiuretic hormone from the posterior pituitary (2).

On the other hand, the loss of water by evaporation through the skin or the respiratory tract leads to a concentration of the plasma electrolytes, which through autonomic receptive elements, »osmoreceptors», stimulates the neurohypophysis to release antidiuretic hormone (5).

The present study was undertaken in order to elucidate, whether quite short exposures to a high ambient temperature effect an antidiuretic response; by keeping the exposure short, and by selecting the rat, an animal with a rather poor ability to sweat, as the experimental animal, it was hoped to avoid a significant loss of water through extrarenal channels.

MATERIAL AND METHODS

Ten conscious albino rats, 5 male and 5 female, were used as the experimental animals. They were first trained to the experi-

mental procedure. Before each experiment the rats were fasted for 5 hours. In order to bring the animals to a standard state, they were given a prehydrating dose of water, 2.5 per cent of body weight, by gavage 1½ hrs before the actual experiment. At the beginning of each experiment, they were given a hydrating dose of water, 5 per cent of body weight. The rats were placed each in a metabolism cage and the voluntarily passed urine was collected into graduated cylinders. The amount of urine was recorded at 15 min. intervals. The urine was collected for 3 hrs. In 25 experiments the cages and their stands were moved into a Finnish »Sauna»-bath exactly 45 minutes after the beginning of the experiment. The rats were kept at +75°C for 5 minutes.

The loss of weight during the exposure to heat was found to be quite insignificant. It was also controlled that evaporation of urine from the funnels used in the experiments was so small that it did not affect the results.

A similar number of control experiments on the same rats were performed at room temperature.

RESULTS

The volumes of urine passed during each 15 min. period are given as per cent of the volume of the hydration dose. The mean

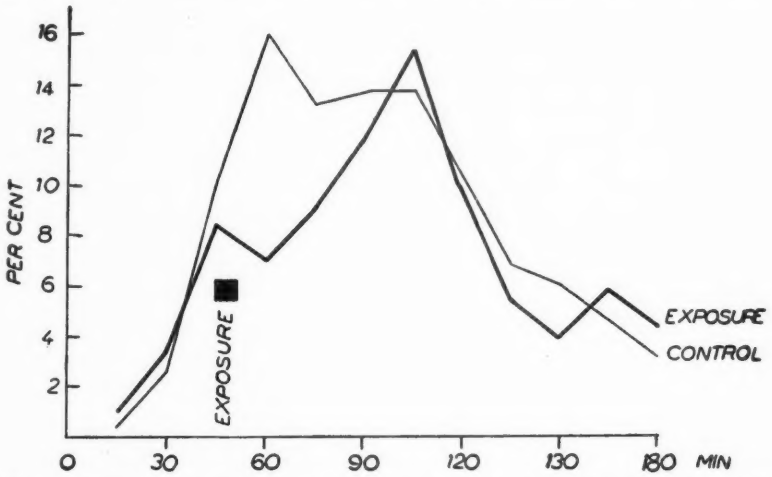

Fig. — The average urine flow as per cent of the hydration dose collected at 15 min. intervals. Thick line: 5 min. exposure to +75°C. Thin line: controls.

values of all the heat-experiments and of the controls are shown in the Fig. It appears that the exposure to the heat delays the maximum diuresis by 45 min., and that the amounts collected 15 and 30 minutes after the beginning of the exposure are considerably smaller than at a corresponding time in the control experiments.

The difference between the two curves could be confirmed statistically, with the aid of the t-test (4). The difference between the volumes collected during the first 15 min. after the exposure and those in the corresponding controls proved significant at the $p < 0.001$ level ($t = 4.90$), and during the second 15 min. period the difference was significant at the $p < 0.05$ level ($t = 2.12$). For the volume collected during these two periods together, the difference was significant at the $p < 0.001$ level ($t = 4.76$).

DISCUSSION

The present results indicate clearly that the exposure of rats during 5 minutes to +75° C ambient temperature immediately diminishes their water diuresis. This agrees with the results of previous studies with milder and more protracted exposures to heat. The present results also show that such a short influence of external heat produces a rather notable reduction of diuresis for about 0.5 hrs. afterwards, although no significant loss of weight through evaporation could be noticed.

The results do not indicate, whether the *action of heat* has operated through some vasomotor disturbances or through the neurohypophyseal mechanism described above. However, the previous studies of Bonvallet et al. (1, 2) on neurohypophysectomized dogs support the possibility of a release of pituitrin. According to Rydin and Verney (3), *emotional stress* causes an inhibition of water diuresis in the dog through the release of antidiuretic hormone. In the present experiments, the animals clearly showed signs of discomfort during exposure, and this may also have contributed to the antidiuresis.

SUMMARY

1. The effect on diuresis of an exposure to +75° C ambient temperature for 5 min. was studied in hydrated rats.

2. The exposure, which did not cause any significant loss of

weight through extrarenal channels, produced a marked diminution in the urine flow, beginning immediately on exposure and lasting for appr. half an hour afterwards.

3. In an analogy to previous investigations, the antidiuresis was interpreted as being due to a release of the antidiuretic hormone.

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FROM THE DEPARTMENT OF SEROLOGY AND BACTERIOLOGY AND FROM THE SECOND MEDICAL CLINIC, UNIVERSITY OF HELSINKI

DECREASED EFFECT OF AUREOMYCIN IN THE PRESENCE OF DIFFERENT SERA

by

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(Received for publication April 4, 1952)

It is well-known that several antibiotics lose their effect in the presence of serum (1). This has also been proved with aureomycin (2).

The writers' own investigations have been concerned with the question of whether this type of influence on the activity of aureomycin varies in different sera.

Method. — *Escherichia coli* was used as the test bacterium and Frenckel's solution, with 1/2 per cent glucose and 0.12 per cent bromthymol blue, as the culture medium. 10 ml of the culture medium was inoculated with 0.12 ml of a 24 hours' culture of the test strain in Frenckel's solution, after which 2.5 ml of the serum to be tested was added. The sera were taken partly from persons suffering from different diseases, partly from healthy persons. A series was obtained by pipetting into every tube 0.5 ml of the inoculated substrate. In addition, 0.5 ml of aureomycin was pipetted into the first of these tubes, a quantity of 0.5 ml being transferred from it into the second, and so on. The incubation time was 24 hours at 37° C. The limit of growth was indicated by the changing of the indicator colour.

Results. — In the control series, using Frenckel's solution without serum, 0.8 μg/ml of aureomycin sufficient to prevent bacterial growth.

For the different sera, totalling 20, the corresponding amounts of aureomycin varied between 6.2 and 3.1 μg/ml. These fluctuations are fully within the titration margins of error.

Experiments were also made with 5 sera using *Staphylococcus aureus haemolyticus* as the test bacterium. No significant fluctuations were observed in the inhibition, as far as the effects of aureomycin is concerned.

SUMMARY

No individual variations in the capacity of different sera to reduce the effect of aureomycin were observed.

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FROM THE DEPARTMENT OF SEROLOGY AND BACTERIOLOGY AND THE DEPARTMENT OF BOTANY, UNIVERSITY OF HELSINKI

DESOXYRIBONUCLEASE IN FUNGI

by

REINO MÄKITALO, HILLEVI ALANEN, and NICKEN MALMSTRÖM

(Received for publication April 8, 1952)

Tillet and his collaborators 1934 were the first to isolate streptokinase, a fibrinolytic enzyme, from streptococcal extracellular enzymes, while Sherry *et al.* (15) 1948 isolated streptodornase, desoxyribonuclease, whose depolymerising property affects the nucleoproteins.

Mac Charty (2), in 1946, isolated desoxyribonuclease in crystals from the beef pancreas, and it was also found to occur in certain types of pneumococci.

We have found no data concerning the occurrence of desoxyribonuclease in the plants, and the object of the investigation reported in this paper was to study desoxyribonuclease activity in Finnish fungi.

MATERIAL AND METHOD

For the purposes of the study the fungi were gathered in the vicinity of Helsinki (Regio Nylandia). The fresh fungi (1 part) were ground by hand in a mortar and extracted with saline (9 parts). The suspension was left to stand at 37° C for an hour and was then centrifuged for 10 minutes at 3 000 r.p.m. The solution obtained was stored in a deep-freezer (—20° C). For the measuring of the desoxyribonucleases of our extracts the method of Kurnick

The editor regrets that, for technical reasons, certain inaccuracies in the article *Desoxyribonuclease in Fungi*, by Reino Mäkitalo, Hillevi Alanen, and Nicken Malmström, could not be corrected before the number of *Annales Medicinae Experimentalis and Biologiae Fenniae* which contained the article was printed. The corrections have been made in the offprints of the article.

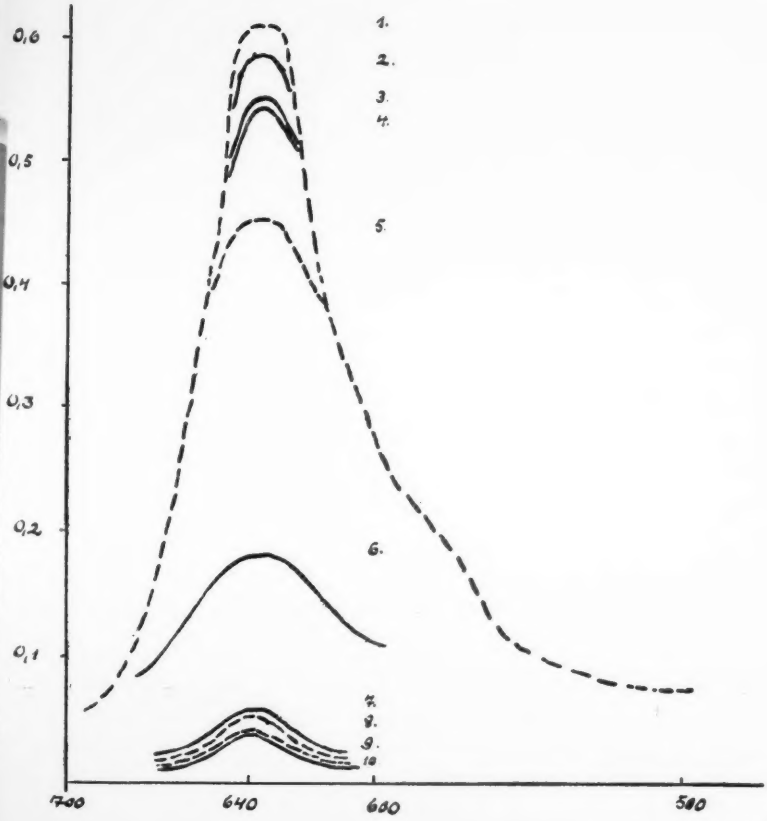

Fig. 1. — Diagrammatic illustration of three fungus extracts of different degrees of activity with and without heating, performed with the Beckmann spectrophotometer at maximal absorption.

was selected. It is based upon the binding capacity of desoxyribonucleic acids of methyl green (10).

A standard solution was prepared, composed as follows:

Methyl green	3.75 cc
DNA aqueous solution 1 per cent	3.75 »
$MgSO_4$ 0.1-M solution	7.50 »
Collidine buffer	50.00 »
Aq. dest. + toluene	35.00 »
	100.00 cc

The strength of the methyl green solution was 0.065 per cent, which was prepared to a 0.02-M acetate buffer with a *p*H of 4.2. The solution was extracted several times with $CHCl_3$, until no colour remained in the chloroform.

DNA was isolated from the thymus of the calf as an Na-salt (14) and stored dry. For use, a 1 per cent aqueous solution was prepared which could very well be kept in a refrigerator. The DNA content of the standard solution amounted to 0.04 per cent. The Mg-ion acted as activator of the enzyme. The collidine buffer (2, 4, 6-collidine l. s-collidine, 0.05-M solution, *p*H 7.6) was used in buffering the ultimate *p*H 7.5 of the standard solution. A drop of toluene was added to prevent microbial contamination. The standard solution thus prepared was allowed to stand for 24 hours at room temperature, whereafter the Beckmann spectrophotometer was used to measure maximal absorption (640 mμ). The extinction coefficient in the maximal absorption region was for our standard 0.612.

TABLE 1

N:o (Fig. 1)	Curve	$E_{640\ m\mu}$
	Positive controls:	
9.	Varidase	0.040
8.	Streptococci broth	0.047
	Unheated act. fungus extracts:	
10.	Clitocybe infundibuliformis	0.039
7.	Mycena galericulata	0.053
6.	Stropharia aeruginosa	0.178
	Negative controls:	
1.	Standard solution	0.612
5.	» » + NaCl (10:1)	0.449
	Heated active fungus extracts:	
2.	Clitocybe infundibuliformis	0.588
4.	Mycena galerfculata	0.543
3.	Stropharia aeruginosa	0.553

Table 1 presents readings determined with the Beckmann spectrophotometer, performed on three fungus extracts of different degrees of activity with and without heating, as well as the controls.

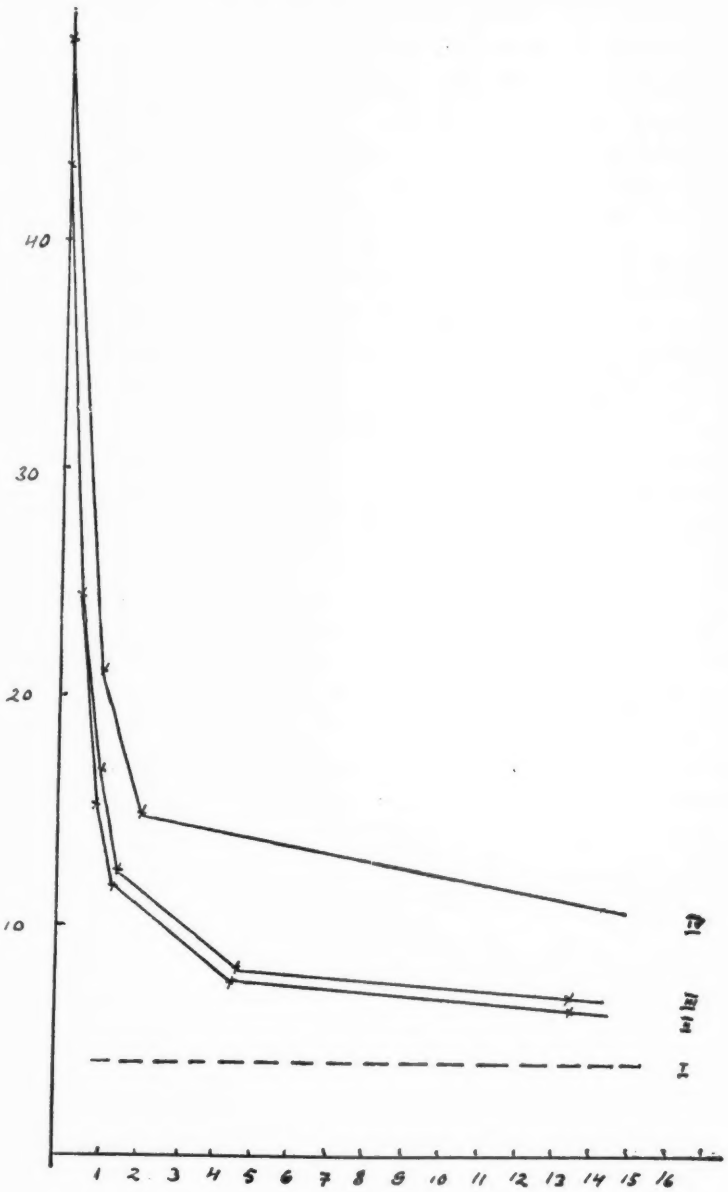

Fig. 2. — A' control study on the assumed spontaneous ability of fungus extracts to reduce the colour of the indicator solution. No such phenomenon occurred.

I. H_2O
II. NaCl-control
III. *Lactarius torminosus*
Cantharellus cibarius
Mycena galericulata
IV. *Lactarius turpis*

The activity of the fungus extracts was studied by adding the extract to the standard solution at a ratio of 10:1 (5 cc of standard solution and 0.5 cc of fungus extract). The reading was performed in 18 to 24 hours with Evely's photoelectric colorimeter, using a 640 mμ filter. This was a quick and efficient method in segregating the positive responses from among the great number of negative specimens. The extracts which proved to be positive were then subjected to a closer study by the Beckmann spectrophotometer and compared to the controls.

As positive control a filtered broth culture of streptococci was used as such, as well as a highly purified streptokinase — streptodornase preparation, viz. Varidase (Lederle Laboratory) 1250 units instead of the fungus extracts. As negative control saline was used instead of the enzyme, and in the same proportion. All active fungus solutions were found to be thermolabile, since when heated (for 10 min. in a 100 C° water bath), their desoxyribonuclease effect was lost.

The colour-reducing quality of the fungus extracts was controlled by omitting DNA from the standard solution used, whereupon the solution rapidly began to lose colour. Accordingly in the same proportion as before saline was added to one tube of methyl green solution, and another fungus extract instead of saline. The rapidly ensuing fading of the colour was observed by means of a colorimeter in both tubes, and it was found that this fading progressed with approximately the same rapidity in both. A graphic plotting of the phenomenon shows that the fungus extracts as such are incapable of reducing the colour of methyl green.

RESULTS

Table 2 presents the fungi examined, in their alphabetical arrangement, classified into four systematic groups. The grouping into active, fairly active and inactive extracts was performed with the help of Evely's photoelectric colorimeter. The »active» extracts reacted like the positive controls used.

TABLE 2

Name and Grouping of the Fungi		
Inactive	Active	
	Fairly Active	Pronounced Activity (corresp. to Posit Contr.)
HYMENOMYCETES		
Nomenclature principally according to Karsten (7, 8), supplemented with other authors listed in the bibliography (1, 5, 6, 9, 11, 12)		
		Amanita muscaria (L) Fr. forma aureola (Kalchbr.)
	Amanita porphyria (Alb. & Schw.) Fr.	
Amanitopsis vaginata (Bull.) Roz.		
	Armillaria mellea (Vahl.) Fr.	
Bjerkandera irregularis (Scop.)Karst. = Leptoporus amorphus (Fr.) Quél.		
Boletus bovinus L.		
» edulis Bull.		
	Boletus luteus L.	
» scaber Bull. var niveus Fr.		
» subtomentosus L.		
» variegatus Schwartz.		
» versipellis Fr.		
Calodon cyathiformis (Schaeff.) Quél.		
		Cantharellus auranthiacus (Wulf.) Fr.
		» cibarius Fr.
Cantharellus tubiformis Fr.		
» umbonatus (Pers.) Fr. = C. muscoides (Wulf.) Karst.		
Clitocybe clavipes(Pers.) Fr.		
» connata(Schum.)Fr		
Clitocybe ditopus Fr.		
» dicolor (Pers.) L.		
		Clitocybe infundibuliformis (Schaeff.) Fr.
» gilva (Pers.) Fr.		
» odora (Bull.) Fr.		

TABLE 2 (Cont.)

Name and Grouping of the Fungi		
Inactive	Active	
	Fairly Active	Pronounced Activity (corresp. to Posit. Contr.)
	Clitopilus orcella (Bull.) Fr.	
Clitopilus prunulus (Scop.) Fr.		
Collybia butyracea (Bull.) Fr.		
		Collybia confluens (Pers.) F
» dryophila (Bull.) Fr.		
» maculata (Alb. & Schw.) Fr.		
Coprinus comatus (Schum)Fr.		
Cortinarius alboviolaceus Fr.		
» armillatus Fr.		
» bolaris (Pers.) Fr.		
	Cortinarius camphoratus Fr.	
» pholideus Fr.		
	» sangvineus(Wulf) Fr.	
	» semisangvineus Fr.	
	Fomitopsis pinicola (Fr.) Karst. = Ungulina marginata (Fr.) Pat.	
Galera tenera (Schaeff.) Fr.		
Gomphidius glutinosus (Schaeff.) Fr.		
» roseus Fr.		
	Hansenia zonata (Fr.) Karst. =Coriolus zonatus (Fr.) Quél.	
		Hebeloma crustuliniforme (Bull.) Fr.
		Hydnum corrugatum Fr.
Hydnum repandum L.		
» rufescens Pers.		
	Hygrophorus agathosmus Fr.	
		Hygrophorus olivaceoalbus Fr.
Hypoloma candolleanum Fr.		
	Inocybe asperospora Quél.	
	Lactarius camphoratus (Bull.) Karst.	

TABLE 2 (Cont.)

Name and Grouping of the Fungi		
Inactive	Active	
	Fairly Active	Pronounced Activity (corresp. to Posit. Contr.)
	Lactarius glyciosmus Fr. sensu Knauth & Neuhoff = confusus Lundell.	
	» helvus Fr.	
	» rufus (Scop.) Fr.	
		Lactarius subdulcis (Pers.) Fr. sensu Karst., Lundell etc. = L. thejogalus (Bull.) Fr. s. Knauth & Neuhoff.
		Lactarius torminosus (Schaeff.) Fr.
		» turpis (Weinm.) Fr.
	» trivialis Fr.	
	» vietus Fr.	
Lenzites betulina (L.) Fr.		
Lenzitina saepiaria (Schaeff.) Karst.		
Lepiota amianthina (Scop.) Fr		
» carcharias (Pers.) Fr.		
	Lepiota cristata (Alb. & Schw.) Fr.	
Marasmius scorodonius Fr.		
		Mycena galericulata (Scop.) Fr
	Mycena metata Fr.	
Mycena pura (Pers.) Fr.		
	» rosella Fr.	
» vulgaris)Pers.) Fr.		
Naematoloma capnoides (Fr.) Karst.		
» sublateritium (Schaeff.) Karst.		
	Naucoria escharoides Fr.	
Nolanea hirtipes (Schum.) Fr.		
Paxillus atrotomentosus (Batsch.) Fr.		
» involutus (Batsch.) Fr.		

TABLE 2 (Cont.)

Name and Grouping of the Fungi		
Inactive	Active	
	Fairly Active	Pronounced Activity (corresp. to Posit. Contr.)
Panaeolus campanulatus L. Fr.		
Pholiota mutabilis (Schaeff.) Fr.		
	Pleurotus mitis (Pers.) Fr.	
Pluteus cervinus (Schaeff.) Fr.		
	Polyporus ovinus (Schaeff.) Fr.	
Psalliota arvensis (Schaeff.) Fr.		
Psathyrella disseminata (Pers.) Fr.		
Psilocybe uda (Pers.) Fr.		
Russula claroflava Grove		
» decolorans Fr.		
	Russula emetica (Schaeff.)Fr.	
	» fragilis (Pers.) Fr. =R. Mairei Singer (Lange 1940)	
	Russula paludosa Britz. = R. elatior Lindbl.	
	Stropharia aeruginosa (Curt.) Fr.	
Stropharia depilata (Pers.)Fr.		
» semiglobata (Batsch.) Fr.		
Tricholoma flavobrunneum Fr.		
Tubaria furfuracea (Pers.) W.Sm.		
	GASTEROMYCETES	
	Nomenclature according to Th. Fries (4)	
	Lycoperdon perlatum Pers. = L. gemmatum Fr.	
	Lycoperdon pyriforme Pers.	

TABLE 2 (Cont.)

Name and Grouping of the fungi		
Inactive	Active	
	Fairly Active	Pronounced Activity (corresp. to Posit. Contr.)
	DISCOMYCETES	
	Nomenclature according to W. Migula (14)	
	Otidea leporina (Batsch.) Fuck.	
Otidea onotica (Pers.) Fuck.		
	MYXOMYCETES	
	Nomenclature according to Rob. Fries (3)	
Lycogala Epidendrum(L.) Fr.		

SUMMARY

The desoxyribonuclease activity of saline extracts in Finnish fungi was studied. Of 100 extracts 42 per cent were found to be active. 12 per cent of the total were strongly active and 58 per cent failed to produce any reaction. All active extracts were found to be thermolabile.

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FROM THE DEPARTMENT OF FORENSIC MEDICINE, UNIVERSITY OF HELSINKI

THE FORENSIC-MEDICAL IMPORTANCE OF THE QUANTITY OF BARBITURIC ACID DERIVATIVES IN URINE

I

A METHODICAL STUDY

by

ANTTI ALHA

(Received for publication May 5, 1952)

Owing to the greatly increased use of barbiturates, their toxicologic investigation has during recent years occupied a more and more important part in forensic-chemical examination, as well in Finland as in other countries. In addition to suicides, attempted suicides, and cases of accidental poisoning, a toxicologist may, for instance, be consulted in cases where barbiturates are used as narcotics. According to Finnish legislation the driving of motor and other vehicles under the influence of narcotics is a penal offence (since August 1, 1950). Whenever such a suspicion has arisen toxicologic investigation of barbiturates in excretions or body fluids of the party concerned is to be considered a necessary forensic-medical measure.

Besides the identification of barbiturates, attempts must be made to estimate the quantity taken, a factor which may greatly influence the judgement of the case, *i. e.*, barbiturates must be determined quantitatively if possible.

Trying to find out whether living persons are under the influence of barbiturate, examination of urine comes perhaps first in question.

On the basis of a urine finding it is, however, in many cases difficult, according to what we know, to draw any definite

conclusions concerning the quantity taken (13). Depending on the barbiturate, its fate in the organism shows great variations. Possibly only few of the barbiturates, such as barbital and phenobarbital, are secreted into urine as such (12). Others decompose in certain parts of the organism, such as amytal and evipan, which decompose in the liver (9). Thus, barbitals may appear in urine partly unchanged (2), partly more or less decomposed (5), etc., and some of them disappear in the organism so completely that not even traces of them can be detected in the urine (11). It has been stated that, used in therapeutic doses, barbital and phenobarbital are the only barbitals consistently detectable in urine (6, 12).

In order to draw conclusions concerning barbiturates secretable into urine, it is necessary to have a method by which it is possible to isolate them fairly quantitatively.

Toxicologic examination of organ samples can be divided into 3 stages: 1. Extraction. 2. Purification of crude material. 3. Identification.

In the toxicologic examination of urine at the Department of Forensic Medicine of the University of Helsinki the method of Stas-Otto (8) and the lead acetate method of Fischer (3) have been generally employed at the extraction stage. For the purification of the crude material extractable into ether from an acid solution and containing the potential barbituric acid derivatives, filtration in a filter tube through MgO and animal charcoal according to Fischer (4) has been employed during the two last years. In addition to these, a number of methods for the isolation of barbituric acid derivatives from urine has been presented in the relevant literature. We have, in Finland, no information based on experiments about the quantitativeness of results obtained with different methods.

The object of this investigation is to assess the applicability of certain extraction and purification methods to the quantitative isolation of barbituric acid derivatives from urine.

An essential part of the extraction is the precipitation of biologic material. In this investigation 4 methods were employed for the precipitation: 1. Stas-Otto's method (8). 2. The acetone method (1). 3. The lead acetate method (3). 4. The alkali hydroxide method (10).

The extraction was carried out with ether alcohol (10), in all cases from a quantity equivalent to 50 ml of urine.

The purification of crude material was carried out with 3 methods parallelly: 1. Purification with MgO and animal charcoal (4). 2. Purification with animal charcoal (14). 3. Purification with a mixture of $CaCO_3$ and animal charcoal (10).

The methods were carried out in detail as follows.

EXTRACTION

1. Stas-Otto's Method. — 150 ml of urine was made clearly acid to litmus with saturated (ca. 20 %) tartaric acid solution and evaporated on water bath to ca. 15 ml. This was extracted with a total quantity of 200 ml of absolute alcohol in several aliquots with simultaneous heating on water bath. After cooling, the alcohol solution was filtered and distilled on 60° C water bath in vacuum to a sirup consistency. The residue was suspended with ca. 200 ml of absolute alcohol and was allowed to stand over night. After this the mixture was filtered, distilled, precipitated with alcohol etc., so many times that no precipitate formed with alcohol at standing over night. In most cases 3 alcohol precipitations were enough. The last distillation residue was dissolved into 150 ml of distilled water with simultaneous careful washing of the flask. After careful mixing the water solution was divided by means of volumetric flasks into three parallel samples of 50 ml.

2. The Acetone Method. — In this method the pre-treatment was carried out in the same way as above but by using acetone. In most cases only one acetone precipitation was necessary. The distillation residue was dissolved into 120 ml of distilled water with simultaneous addition of 20 g of $(NH_4)_2SO_4$. The solution was filtered, the flask and filter paper washed with 10 % $(NH_4)_2SO_4$ solution so that the quantity of filtrate including wash water was 150 ml. The water solution was divided into 3 parts for parallel experiments.

3. The Lead Acetate Method. — Into 50 ml of urine 5 ml of 0.5-n solution of lead acetate was added. The mixture was made clearly acid to litmus with acetic acid and was allowed to stand for half an hour. After this it was filtered. The precipitate was washed several times with a small quantity of water containing acetic acid. 3 parallel experiments were made.

4. The Alkali Hydroxide Method. — 50 ml of urine was made just alkaline to litmus with sodium hydroxide solution and filtered immediately and the precipitate was washed with slightly alkaline water. After this the solution was made clearly acid to litmus with hydrochloric acid. Three parallel experiments were made.

All water solution samples thus obtained were shaken with ether alcohol 20:1. According to experience a small amount of alcohol in ether reduces the formation of an emulsion frequently appearing in shaking. Peroxide and water free ether (Orion) and absolute alcohol (State Alcohol Company) were used. When a 75 ml sample of ether alcohol was allowed to stand with calcined, colorless $CuSO_4$ for 24 hours, no change of color

could be observed in it; accordingly the mixture could be considered waterless. The shaking was performed three times, at each time with a 25 ml aliquot for 1 min. The aliquots were brought together and the 75 ml of ether alcohol extract were washed with water and dried over night with 1 g of anhydrous Na_2SO_4 (BDH). After that the extracts were filtered in filter tube. As such a glass tube 25 cm in length was used. The inner diameter of its 5 cm long lower part was 4 mm and the inner diameter of the upper part 10 mm. The upper end was widened to a funnel. At the junction of the upper and lower parts a small stopper wad was placed and upon it ½ g of Na_2SO_4. — Turfitt (14) has employed a rapid method in which he allowed the ether extract to stand with Na_2SO_4 for 2 minutes for drying and after that filtered it through a filter tube containing Na_4SO_4. In preliminary tests, however, the drying proved insufficient. For this reason an examination was made in order to get a rough idea of the efficiency of Na_2SO_4 drying. 75 ml of ether alcohol saturated with water was treated with 1 g of Na_2SO_4 for different periods of time 1. by shaking, 2. by allowing to stand, after which it was filtered through a filter tube containing Na_2SO_4 and shaken with waterless $CuSO_4$. The time required for the $CuSO_4$ to turn clearly bluish was recorded. The results were as follows:

Time of Treatment with Na_2SO_4		$CuSO_4$ Colored
Shaking	2 minutes	At once
»	5 »	Ca. 1 minute
»	10 »	2—3 »
Standing	4 hours	Ca. ½ »
»	18 »	2—3 »
»	72 »	2—3 »

The results show that with 18 hours' standing a degree of dryness is obtained which is possible with Na_2SO_4 under the conditions described above. With regard to rapid methods this is also obtainable with 10 min. shaking.

The filtration of extracts was conducted into 100 ml glass bowls of 25—35 g constant weight which was controlled from time to time. The solvents were evaporated on water bath after which the bowls were kept in an drying cabinet at 70° C for an hour and put into desiccators for night. »Blaugel» was used as drying medium. The weighing of the crude material thus obtained into bowls took place with the automatic Galileo Sartorius balance at the accuracy of 0.1 mg. It can be mentioned that in order to maintain the constant weight of the bowls they were treated with neither alkali nor acid but washed with water, alcohol and ether.

PURIFICATION

For purification the crude materials in bowls were dissolved into ether alcohol and drawn by suction into vacuum tube through filter tubes containing an absorptive packing.

1. Purification with MgO-Charcoal. — The lowermost layer in the filter tube was ca. 0.2 g of charcoal (Merck) and upon it there was ca. 0.2 g of MgO (Kahlbaum).

2. Purification with Charcoal. — The ether alcohol solution was shaken with ca. 0.2 g of charcoal for 2 min. and treated in filter tube containing the same amount of it.

3. Purification with a Mixture of $CaCO_3$ and Charcoal. — A mixture was used which contained equal parts by weight of $CaCO_3$ (Merck) and charcoal. The ether alcohol solution was shaken with ca. 0.2 g of the mixture for 2 min and was treated in filter tube whose packing consisted of ca. 0.2 g of the mixture, likewise.

The quantity of ether alcohol used in the purification of each crude material was, including washing etc., ca. 75 ml. The ether alcohol extract was treated and finally weighed in the same way as described above for crude material ether extract. — It can be mentioned that the wad stopper supporting the packing must be packed very hard so that no absorption substance should come into the filtrate in the suction. Before filtration the packing must be washed with the solvent.

CONTROL INVESTIGATION OF URINE

For control, a series of examinations was carried out with the urine of 10 healthy persons, who had taken no medicine. The samples were first allowed to stand for 5—10 days at room temperature in order to be clearly in a state of foulness. The *p*H was at the beginning of the investigation between 5—6.5. The results are shown in Table 1. It can be seen that the smallest yields of crude material are obtained with the NaOH precipitation and $(CH_3CO_2)_2Pb$

TABLE 1

THE MAXIMUMS OF SINGLE EXPERIMENTS AND THE MEANS OF ALL RESULTS OBTAINED WITH DIFFERENT PRECIPITATION AND PURIFICATION METHODS WITH 10 URINES IN THE CONTROL INVESTIGATION

	Crude Materials		Purified Yields					
			MgO-Charcoal		Charcoal		$CaCO_3$-Charcoal	
	Maximum mg	Mean mg	Maximum mg	Mean mg	Maximum mg	Mean mg	Maximum mg	Mean mg
Stas-Otto's method	34.8	20.0	15.4	7.8	8.5	3.6	14.9	10.0
Acetone »	20.4	10.7	8.7	2.8	4.7	1.6	10.9	4.3
$(CH_3CO_2)_2$ Pb »	12.0	6.9	4.4	1.6	5.0	1.4	6.1	2.8
NaOH »	9.5	4.6	1.5	0.6	3.0	1.0	3.9	1.7

precipitation methods, and that the yield of purification obtained from their crude material is clearly smaller with the charcoal and MgO-charcoal methods than with the $CaCO_3$-charcoal method. In addition in the vacuum filtration of the latter method a strong tendency for splashing was noticed.

In the preliminary tests it was found that when the NaOH precipitation was used, the *p*H of urine has an influence on the magnitude of the crude material. The following experiment illustrates this point:

*p*H of Urine at Filtration	Yields of Crude Material, 2 Parallel Experiments
4.4	8.9 and 8.0 mg
7	3.1 » 3.5 »
8	3.1 » 3.8 »
10	3.7 » 3.8 »
11—12	7.8 » 9.0 »

In strongly alkaline urine some acid substance extractable into ether alcohol is obviously liberated through hydrolysis immediately.

EXPERIMENTS WITH DERIVATIVES OF BARBITURIC ACID

12 derivatives of barbituric acid commonly known in Finland were obtained for the investigation. From these nembutal was as Na salt and phanodorm as Ca salt which were isolated as free acids. All the preparations were recrystallised from water by simultaneous use of charcoal for purification. The products were dried with P_2O_5 in desiccator. Table 2 shows the preparations used in the investigation and their melting points determined both with Roth's apparatus and Kofler's micro apparatus for melting point determination. The melting points are also presented according to Kofler (7). In Roth's apparatus the lower limit of melting point is determined when a beginning sinking is noticed in the capillary. In Kofler's apparatus the temperature at the appearance of potential condensation droplets was also taken into consideration.

The first examinations with derivatives of barbituric acid were carried out in order to establish the influence of technical procedures of the investigation on the quantitativeness of results. About 20 mg of derivatives of barbituric acid weighed accurately were dissolved into 75 ml of ether alcohol. The ether alcohol solutions were treated according to the general procedure used in the investigation as follows: 1. Evaporation to dryness directly and 2. after Na_2SO_4

TABLE 2

DERIVATIVES OF BARBITURIC ACID AND THEIR MELTING POINTS, C°.—C.DR.= CONDENSATION DROPLETS

Substituents	Melting Points acc. to Kofler	Melting Points Determined	
		Roth's Apparatus	Kofler's Apparatus
1. 5-ethyl-5-n-butyl-. (Soneryl)	126	123 —125	123—124
2. 5-ethyl-5 (1-methylbutyl)-. (Nembutal)	129	128 —130.5	129—131. C.dr. 128
3. 5-allyl-5-isopropyl-. (Alurate)	142	139 —141	141 —141.5
4. N-methyl-5-methyl-5-cyclohexenyl-. (Evipan)	146	143 —145	143.5—145
5. 5.5-dipropyl-. (Proponal)	148	147 —149	147 —148. C.dr. 145
6. 5-ethyl-5-isoamyl-. (Amytal)	156	152 —155	156 —158.5. C.dr. 153
7. 5-ethyl-5-cyclohexenyl-. (Phanodorm)	173	173.5—175	171 —173. C.dr. 156
8. 5.5-diallyl-. (Dial)	174	170 —172	172.5—173. C.dr. 165
9. 5-ethyl-5-phenyl-. (Phenobarbital)	174	175 —177	172.5—174.5. C.dr. 135
10. 5-allyl-5-β-bromallyl-. (Vesperone)	—	172.5—174.5	173 —176. C.dr. 151
11. N-methyl-5-ethyl-5-phenyl-(Prominal)	179	176	174 —177. C.dr. 147
12. 5.5-diethyl-. (Barbital)	190	188 —192	189. C.dr. 185

treatment. 3. According to different purification methods. With all derivatives of barbituric acid 3 parallel experiments were made in each treatment. The weight of evaporation residues was expressed as per cent of the quantity weighed. The means of all percentages obtained each treatment were calculated. The results were as follows:

Treatment	Mean
Direct evaporation	97.5 %
Na_2SO_4 treatment	96.5 »
Purification:	
MgO-charcoal method	94.5 »
Charcoal »	95.5 »

it can be seen that on direct evaporation a part of derivatives of barbituric acid disappears, which is understandable owing to their

TABLE 3

THE ISOLATION OF DERIVATIVES OF BARBITURIC ACID DISSOLVED INTO URINE WITH

	Urine 1, *p*H 5.42							
	Control				Barbital 20 mg			
	Crude Material	Purif. with MgO-C	Crude Material	Purif. with C	Crude Material	Purif. with MgO-C	Crude Material	Purif. with C
Stas-Otto's method	25.0	12.6	27.4	6.5	47.7	29.7	46.3	24.1
$(CH_3CO_2)_2$ Pb »	9.6	2.0	9.3	2.8	28.8	19.5	28.4	17.9
NaOH »	5.6	1.4	5.8	1.2	20.6	19.2	21.8	18.6

sublimatory character. Possibly also a part, although a very slight part, is lost with Na_2SO_4. In testing the purification methods a great scattering was found between the parallel experiments of the $CaCO_3$-charcoal method. This is obviously due to the splashing tendency of ether alcohol when the solution is drawn by suction into the vacuum tube as was mentioned above. For this reason the method was discarded. In the case of the purification methods dealt with the loss together with that caused by evaporation is to be regarded as about 5 %. Fischer (4) has mentioned a loss of 5 % in the MgO-charcoal method.

For the next a control investigation was carried out by shaking the derivatives of barbituric acid from water solutions following out the general procedure. About 20 mg of derivatives were dissolved into 50 ml of water. 3 parallel experiments were made with the preparation and the percentage of evaporation residues was calculated as above. The results were as follows:

Soneryl	91.4 %	Phanodorm	93.5 %
Nembutal	93.3 »	Dial	94.2 »
Alurate	93.5 »	Phenobarbital	97.9 »
Evipan	94.5 »	Vesperone	98.0 »
Proponal	94.0 »	Prominal	98.5 »
Amytal	93.1 »	Barbital	92.8 »
Mean	94.6 »		

3

WITH 3 PRECIPITATION AND 2 PURIFICATION METHODS. THE DATA IN MG

Urine 2, *p*H 5.88								Urine 3, *p*H 5.69							
Control				Nembutal 20 mg				Control				Phanodorm 20 mg			
Crude Material	Purif. with MgO-C	Crude Material	Purif. with C	Crude Material	Purif. with MgO-C	Crude Material	Purif. with C	Crude Material	Purif. with MgO-C	Crude Material	Purif. with C	Crude Material	Purif. with MgO-C	Crude Material	Purif. with C
21.5	7.5	25.6	2.9	35.3	23.3	36.7	22.1	32.2	15.4	25.5	8.5	57.2	46.4	58.6	27.7
8.8	3.6	10.0	1.8	34.8	25.4	32.9	23.7	12.0	4.4	8.3	1.3	29.3	22.7	30.6	22.9
3.6	0.1	2.7	1.0	23.9	19.2	23.1	17.7	4.2	1.5	7.9	3.0	27.7	23.0	31.7	24.3

Loss of weight at evaporation and Na_2SO_4 drying is a factor influencing the results. Accordingly, over 90 % of derivatives of barbituric acid could be extracted from their water solutions by 3 shakings with ether alcohol.

Experiments with Urine. — Barbital, nembutal and phanodorm were dissolved into urine 20 mg per 50 ml. Series with all the precipitation methods and MgO-charcoal and charcoal purification methods were carried out. It was found that there was a considerable scattering between the parallel results of the acetone precipitation method and that the purified yields could be only about half of the quantity expected. For this reason the method was discarded. The results of other methods are given in Table 3. It is evident that the purified yields of crude materials obtained with the NaOH and $(CH_3CO_2)_2Pb$ precipitation methods give a good picture of the quantity of derivatives of barbituric acid added to urine.

Finally an experiment was made with all the derivatives by using the $(CH_3CO_2)_2Pb$ precipitation and MgO-charcoal purification methods and the same urine whose *p*H was 6.08. Into 150 ml of urine 20 mg per 50 ml of the preparations were weighed with the exception of 10 mg per 50 ml of prominal which did not dissolve. After slight heating and standing for a night each urine sample was divided into 3 parallel experiments. The scattering between parallel experiments was comparatively slight. The means of results obtained in parallel experiments were calculated:

	Crude material	Purified Yield		Crude material	Purified Yield
Control	6.1 mg	0.9 mg			
Soneryl	31.0 »	24.2 »	Phanodorm ..	27.2 mg	21.3 mg
Nembutal ..	34.2 »	24.8 »	Dial	27.6 »	20.1 »
Alurate	26.7 »	19.9 »	Phenobarbital	34.4 »	24.2 »
Evipan	26.2	21.4 »	Vesperone	26.9 »	19.4 »
Proponal	27.1 »	19.8 »	Barbital	25.3 »	19.1 »
Amytal	29.6 »	23.9 »	Prominal.....	18.6 »	11.5 »

Accordingly, there were considerable differences in the weights of both crude materials and purified yields between different derivatives. It is probable that the preparations depending on the substituents absorb different quantities of the impurities in urine. The quantity of these in the purified product cannot be simply estimated, for instance on the basis of control experiment on the same urine. However, a fairly good general picture of the quantity of derivatives of barbituric acid added to urine can be derived from the purified yields.

SUMMARY

The object of this investigation was to study the applicability of certain methods of extraction and purification to the quantitative isolation of derivatives of barbituric acid from the urine of living persons. The precipitation methods investigated were: 1. Stas-Otto's method. 2. The acetone method. 3. The lead acetate method. 4. The NaOH precipitation method. The purification methods were: 1. The MgO-charcoal method. 2. The charcoal method. 3. The $CaCO_3$-charcoal method.

In the investigation it was established that from the precipitation methods the NaOH and lead acetate methods, and from the purification methods the charcoal and MgO-charcoal methods give results which give fairly good general idea about the quantity of derivatives of barbituric acid in urine.

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